MAR 3 1932

Vol. XXV, No. 6 1931

THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

ARTHUR HARDEN

AND

CHARLES ROBERT HARINGTON

EDITORIAL COMMITTEE

PROF. G. BARGER
PROF. V. H. BLACKMAN
MR J. A. GARDNER
SIR F. G. HOPKINS
SIR F. KEEBLE
PROF. W. RAMSDEN
SIR E. J. RUSSELL

CAMBRIDGE UNIVERSITY PRESS
LONDON: Fetter Lane, E.C.4
also
H. K. LEWIS & Co., Ltd., 136, Gower Street, London, W.C.1
CHICAGO: The University of Chicago Press
(Agent for the United States)
BOMBAY, CALCUTTA, MADRAS: Macmillan and Co., Ltd.
TOKYO: Maruzen Company, Ltd.

1931

Price Seventeen Shillings and Sixpence net

PRINTED IN GREAT BRITAIN

The *Biochemical Journal* is conducted by the Biochemical Society and is published by the Cambridge University Press.

This Society has been instituted for the purpose of facilitating intercourse between those biologists and chemists who are interested in the investigation of problems common to both, such as the chemical problems connected with Agriculture, Brewing, Animal and Vegetable Physiology and Pathology, &c. Persons interested in Biochemistry are eligible for election.

Meetings are held at different centres for the communication of papers to the Society.

Members are entitled to receive a copy of the Journal. Further information as to the conditions of membership may be obtained on application to the Hon. Sec., Prof. A. C. Chibnall, Imperial College of Science and Technology, South Kensington, S.W. 7, or to the Hon. Treas., Mr J. Addyman Gardner, 12B Hyde Park Mansions, London, N.W. 1, to whom subscriptions should be sent.

Papers for publication should be sent to Prof. A. Harden, F.R.S., Sunnyholme, Bourne End, Bucks. Communications respecting the printing of the articles, or respecting the purchase of offprints should be addressed to the University Press, Cambridge.

The Journal is issued about every two months and the date at which each paper is received by the editors is printed at the beginning of the paper.

All communications respecting the purchase of copies of the parts or volumes, whether current or back issues, or respecting subscriptions in the case of non-members of the Biochemical Society (£3. 10*s.* 0*d.* net per volume (post free) payable in advance) should be addressed to The Cambridge University Press, Fetter Lane, London, E.C. 4, or to the Hon. Treas., Mr J. Addyman Gardner, 12B Hyde Park Mansions, London, N.W. 1. For prices of back numbers and volumes see list on p. 4 of Wrapper.

Quotations can be given for Buckram binding cases and for binding Subscribers' Sets.

The Cambridge University Press has appointed the University of Chicago Press agent for the sale of the *Biochemical Journal* in the United States of America.

Notice to Contributors.

1. Contributors who are members of the Biochemical Society receive 25 copies of their papers free and can purchase additional copies if notice be given when their proofs are returned. Other contributors may purchase reprints but receive no free copies.

2. Papers forwarded to the Editors for publication are understood not to be offered to any other Journal for prior or simultaneous publication.

3. All communications intended for publication must be type-written.

4. **References.** References to literature should be arranged alphabetically according to authors' names at the end of the communication, each accompanied by the date, title of Journal, volume and page, thus: Ackermann (1912), *Z. Biol.* **59**, 17. In the text the reference should be indicated by the author's name and the date, enclosed in brackets, thus [Ackermann, 1912]. If reference is made to several papers published by one author in a single year, these should be numbered in sequence and the number quoted along with the year both in the text and in the collected references.

5. Chemical formulae should be written, as far as possible, in a single horizontal line.

6. Illustrations and curves accompanying the papers must be carefully drawn, about twice the size of the finished block, on smooth white Bristol boards or ordinary curve paper in Indian ink. Any lettering on these drawings should be lightly inserted in pencil. Further information can be obtained from the Editors.

MSS. for the successive numbers of Vol. 26 should be in the hands of the Editors before the 1st of January, March, May, July, September and November respectively. Contributors are specially requested to arrange references in the manner uniformly adopted in this Journal.

CONTENTS OF VOLUME XXV, No. 6, 1931.

(*All rights reserved*)

PAGE

OBITUARY NOTICE 1849

CXCIX. The formation of bacterial proteases, especially in synthetic media. By R. B. HAINES 1851

CC. The effect of hydrogen ion concentration on the precipitating and protective actions of mixtures of two proteins on colloidal gold and gum benzoin. By R. H. HOPKINS, W. O. KERMACK and W. T. SPRAGG. (With fifteen figures) . . . 1860

CCI. Influence of proteins and protein derivatives on the enzymic hydrolysis of starch by malt diastase. By B. FILIPOWICZ 1874

CCII. Spermine in human tissues. By G. A. HARRISON 1885

CCIII. Studies on the metabolism of animals on a carbohydrate-free diet. III. Effect of alkali on the sensitivity towards insulin of albino rats. By A. HYND and D. L. ROTTER 1893

CCIV. Notes on oil development in the seed of a growing plant. By J. V. EYRE. (With two figures) 1902

CCV. The hydrolysis of concentrated sugar solutions by invertase. By H. A. AUDEN and E. R. DAWSON. (With three figures) 1909

CCVI. Synthesis of *i*-β-hydroxyglutamic acid. By C. R. HARINGTON and S. S. RANDALL. With a note on the dissociation constants. By G. M. RICHARDSON. (With one figure) 1917

CCVII. Study of the blood-calcium following the intravenous injection of calcium salts. By G. D. GREVILLE. (With two figures) 1931

CCVIII. The absorption of water by gelatin. Part VII. The influence of temperature on swelling in acid solutions and the combination of gelatin with hydrochloric, nitric or sulphuric acid. By W. B. PLEASS. (With two figures) . . . 1943

CCIX. A method for determining aluminium in certain aluminium protein compounds. By C. G. POPE 1949

CCX. The body fats of the pig. I. Influence of ingested fat on the component fatty acids. By R. BHATTACHARYA and T. P. HILDITCH 1954

CCXI. The liberation of elementary nitrogen by bacteria. By N. W. BARRITT 1965

CCXII. The chemistry of cooking. I. The chemical changes of carbohydrates in the sweet potato according to various methods of cooking. By O. SINODA, S. KODERA and C. OYA 1973

CCXIII. Studies in the biochemistry of micro-organisms. XXII. The isolation and characterisation of ergosterol from *Penicillium puberulum* Bainier grown on a synthetic medium with glucose as sole source of carbon. By J. H. BIRKINSHAW, R. K. CALLOW and C. F. FISCHMANN 1977

CCXIV. Determination of small amounts of phosphatides and cholesterol in tissues. By M. JOWETT and E. W. LAWSON 1981

CCXV. The phosphatide and cholesterol contents of normal and malignant human tissues. By M. JOWETT 1991

CONTENTS

PAGE

CCXVI. The loss of glucose from dried peas on soaking. By W. M. Clifford . 1999

CCXVII. The enzymic synthesis of proteins with special reference to the action of pepsin. By D. P. Cuthbertson and S. L. Tompsett 2004

CCXVIII. A note on the determination of cystine in proteins by the method of Folin and Marenzi. By S. L. Tompsett 2014

CCXIX. The hemicelluloses of the wood of English oak. I. The effect of the drying of wood on the yield and composition of hemicellulose A. By M. H. O'Dwyer 2017

CCXX. The chemistry of the white rots of wood. II. The effect on wood substance of *Armillaria mellea* (Vahl.) Fr., *Polyporus hispidus* (Bull.) Fr., and *Stereum hirsutum* Fr. By W. G. Campbell 2023

CCXXI. Further observations on the oxidation of lactic acid by brain tissue. By C. A. Ashford and E. G. Holmes. (With seven figures) 2028

CCXXII. The distribution of the vitamin B complex. III. Fruits. By M. H. Roscoe 2050

CCXXIII. The effects of coprophagy in rats deprived of the vitamin B complex. By M. H. Roscoe. (With one figure) 2056

CCXXIV. Oxidation-reduction potentials of *Staphylococcus* cultures. II. Effect of bacteriophage. By L. F. Hewitt. (With two figures) 2068

CCXXV. Synthesis and crystal spacings of certain long-chain paraffins, ketones and secondary alcohols. By S. H. Piper, A. C. Chibnall, S. J. Hopkins, A. Pollard, J. A. B. Smith and E. F. Williams. (With Plate VIII and nine figures) 2072

CCXXVI. The wax constituents of the apple cuticle. By A. C. Chibnall, S. H. Piper, A. Pollard, J. A. B. Smith and E. F. Williams 2095

CCXXVII. The wax constituents of forage grasses. I. Cocksfoot and perennial ryegrass. By A. Pollard, A. C. Chibnall and S. H. Piper 2111

CCXXVIII. The dicarboxylic acid-nitrogen of proteins. With a note on the alcohol-soluble protein from ragi (*Eleusine coracana*). By M. Damodaran . . 2123

CCXXIX. Vitamin A and carotene. VIII. High potency vitamin A concentrates. By T. Moore. (With one figure) 2131

CCXXX. A study of the factors influencing the concentration of complement in the blood. By T. W. B. Osborn. (With three figures) 2136

CCXXXI. On the function of torulin. An *in vitro* effect of antineuritic vitamin concentrates. By N. Gavrilescu and R. A. Peters. (With one figure) . . 2150

CCXXXII. The assimilation of aluminium by the human system. By S. J. Lewis 2162

CCXXXIII. Note on the activation of oxido-reductions by co-zymase. By H. v. Euler and R. Nilsson 2168

CCXXXIV. "Resting" bacteria. By B. R. Sandiford and W. R. Wooldridge. (With three figures) 2172

CCXXXV. The nitrogen metabolism of the lupin seedling. By P. McKie. (With one figure) 2181

CCXXXVI. The basic amino-acids of wool. By A. M. Stewart and C. Rimington 2189

Index 2193

BIOCHEMICAL JOURNAL

ERRATA

VOLUME XXV

Page 1075, 5th para., last line:
for 5·5% *read* 6·1%

Page 1076, 1st para., 7th line:
for 60·5% *read* 61·1%

Page 1076, last para., 5th line:
for foregoing *read* following

OBITUARY NOTICE.

JAMES LORRAIN SMITH (1862–1931).

THE much regretted death, in April of this year, of Professor Lorrain Smith of Edinburgh calls for notice in this *Journal*, on account of his contributions on the chemical side to pathology and physiology. The son of a Free Church Minister in Dumfriesshire, he was born in 1862. He studied with great distinction at Edinburgh University, graduating first in Arts, and then in Medicine in 1889. He then went to study and work in Burdon Sanderson's Laboratory at Oxford, and soon afterwards began research work, along with Haldane, on the physiological action of air vitiated by respiration. This resulted in two well-known papers, followed by an independent one on the general metabolism after thyroidectomy. He was then appointed John Lucas Walker Student in Pathology at Cambridge, and later Demonstrator, under Roy, his time there being, however, interrupted by visits to von Recklinghausen's Laboratory at Strasburg to study histology, and to Bohr's Laboratory at Copenhagen to study methods of blood-gas analysis. In 1894 he was appointed Lecturer in Pathology at Queen's College, Belfast, and Professor in 1901.

It was here that he and Haldane carried out the experiments, first on man, and then on animals, from which they concluded that active secretion of oxygen inwards occurs in the lungs, though it was not known to them at the time that the secretion only occurs in response to the stimulus of want of oxygen in the tissues. They also worked out a method for determining in man the total volume and oxygen-capacity of the blood, this method depending on their discovery that the oxygen-capacity and colouring-power of the blood run parallel. With the new method Lorrain Smith investigated various abnormal conditions of the blood, and found that in the so-called anaemia of chlorosis the volume of the blood is in fact greatly increased, but correspondingly diluted as regards haemoglobin, so that the total haemoglobin in the body is unaltered. Other forms of anaemia were found to be true anaemias, with great diminution of the total haemoglobin; while cases with a high percentage of haemoglobin in the blood were found to have also a high blood-volume. Another of his discoveries about the same time was that oxygen at high partial pressures (even less than one atmosphere) produces pneumonia.

In 1904 he was appointed Professor of Pathology at Manchester and in 1912 to the Chair at Edinburgh. He had meanwhile, owing to his wide knowledge and sympathies, become more and more immersed in University and public affairs, leaving him less time for research. He became greatly interested, however, in the chemical conditions which determine the staining of acid or neutral fats, and the light thus thrown on physiological or pathological processes, and

he continued, along with his colleagues, to work at this subject till his death, making a number of discoveries, published in one paper after another.

In connection with the War he and colleagues in his laboratory introduced the well-known powerful antiseptic "eusol" (Edinburgh University solution) containing hypochlorous acid. They also did valuable work on "trench-foot," and he was probably the first to suggest the use of charcoal in respirators.

As a teacher Lorrain Smith was extremely successful, but perhaps even more so in his contributions to University policy and administration. He also served with distinction on various public bodies, and in 1906 he took a leading part in founding the Pathological Society. In 1909 he was elected a Fellow of the Royal Society, and he received honorary degrees from two universities.

J. S. H.

CXCIX. THE FORMATION OF BACTERIAL PROTEASES, ESPECIALLY IN SYNTHETIC MEDIA.

By RAYMOND BENNETT HAINES.

From the Low Temperature Research Station, Cambridge.

(*Received September 10th, 1931.*)

Brunton and Macfadyen [1889] showed that broth cultures of bacteria, moderately heated to kill the organisms, could bring about digestion of proteins. However, Fermi [1890] was apparently the first conclusively to demonstrate that an active proteolytic enzyme can be obtained from a sterile cell-free extract of the medium in which certain bacteria have been grown. Since that time difference of opinion has existed as to whether it is essential for the elaboration of such enzymes to have present in the medium proteins or protein derivatives. Some authors have reported the presence of enzymes after the growth of bacteria in synthetic media containing amino-acids only, or in some cases ammonium salts only, as the source of nitrogen. Others, on the other hand, have maintained that "organic" nitrogen in the form of peptone or some other complex substance, is required for protease formation. Much of the earlier work seems unreliable when viewed from a modern standpoint, since

(*a*) the concept of p_H, its application to bacteriology and its fundamental importance in enzymic activity had not been elaborated;

(*b*) the significance of vitamins in nutrition was unknown and discordant results with synthetic media may have been due to their presence in the amino-acids or other substances used;

(*c*) the organisms were not washed and consequently organic matter was probably carried over from the stock culture to the synthetic medium.

Bainbridge [1911] showed that pure native proteins are not attacked by bacteria and his results were confirmed and enlarged by Sperry and Rettger [1915]. Diehl [1919] stated that amino-acids are required in the medium for the elaboration of enzymes and he reported a curious specificity according to the amino-acid present. Merrill and Clark [1928], however, found that they obtained gelatinase from organisms growing in a synthetic medium of carefully purified salts, with ammonium chloride as the source of nitrogen, provided that salts of calcium and magnesium were present. No gelatinase was obtained in the absence of these salts, despite good growth. Wilson [1930] could not confirm Diehl's specificity. She criticised Merrill and Clark's results on the ground that poor growth only was obtained in the absence of salts of calcium

and magnesium and concluded that these salts are necessary for the elaboration of enzymes only in so far as they stimulate growth.

Numerous points remain to be settled. It is not evident from Bainbridge's work whether failure of the organism to grow was due to inability to utilise pure protein as a source of nitrogen or to lack of some other essential constituent such as the salts mentioned above. Non-decomposition of the protein may have been due as much to the inability of the enzyme to attack the protein, owing to high dilution or other non-optimal conditions, as to failure of the organism to produce enzyme. It is essential to guard against misinterpreting effects which may be due merely to dilution. For example, in the course of the present work it has been found that by diluting an enzyme preparation which formerly attacked two proteins, it may be made to attack only the one. If this latter dilution had been arbitrarily selected in the first instance a case of specificity might have been assumed. Again, if the proteolytic enzymes are concerned in the synthetic processes of the cell as well as in the degradation processes, it is to be expected that these enzymes will be formed whenever good growth occurs. In view of the conflicting views advanced, this latter point requires further investigation, and it is the main purpose of the present paper to attempt to settle it. It was necessary first of all to make a preliminary study of the formation of proteases in broth culture by the organisms selected (*i.e.* under optimum conditions) by way of control. The work was then extended, as far as possible with the same organisms, to synthetic media of known composition.

Protease formation in broth.

Methods.

A single loopful of an overnight culture of the organism to be studied was inoculated into several hundred cc. of nutrient broth, p_H 7·4, spread in a thin layer in a large Fernbach flask. After incubation, during which it had been repeatedly shaken to ensure aeration of the culture, the broth was centrifuged at high speed for 30 minutes and sterilised by filtration through a Chamberland candle. As the result of preliminary tests 2–5 cc. lots of the filtrate were transferred to tubes containing 5–10 cc. of gelatin, caseinogen or egg-albumin solution. These were then incubated for several days and digestion estimated by the methods outlined below. The gelatin was made up according to the method of Wilson [1930] by dissolving 5 g. gold label gelatin in 100 cc. phosphate buffer on a water-bath at 60°, adding phenol to 0·5 % and adjusting the final p_H at 7·4. An approximately 1 % caseinogen solution was prepared by well stirring the required amount with phosphate buffer containing 0·9 % sodium chloride, filtering and adding thymol solution to 1 %, the final p_H again being 7·4. Egg-albumin was obtained from fresh eggs by the modified method of Hopkins used by Adair and Robinson [1930] and twice crystallised. It was dialysed in collodion sacs until a negative test for sulphates was obtained in the liquid outside the sac. Liquefaction of the gelatin was tested by immersing

the tubes direct from the incubator in ice-water, and comparing the time required for setting with that of a control tube. Caseinogen digestion was indicated by absence of precipitate or turbidity on adding 0·4 cc. of alcoholic acetic acid (Diehl). Controls were here again carried out. Digestion of egg-albumin was estimated by adjusting the p_H of the solution approximately to the isoelectric point, coagulating by immersing for five minutes in boiling water, filtering either through Jena sintered glass filters or washed and tared filter-papers, drying at 100° for 4 hours and weighing.

Results.

Table I is an example of the type of result obtained with broth cultures of the organisms listed.

Table I.

	Gelatin		Caseinogen		Egg-albumin	
Organism	Blank	Exp.	Blank	Exp.	Blank g.	Exp. g.
Proteus 3	set 3 mins.	*not* set 24 hrs.	0	++	0·100	0·097
B. mesentericus	„ 3 „	„ „	0	++	0·098	0·084
Pseudomonas 1	„ 4 „	„ „	0	++	0·098	0·100
Pseudomonas 2	„ 3 „	„ „	0	++	0·088	0·077

In the first gelatin column is given the time of setting of the same mixture of enzyme and gelatin as in the second column, but heated to 100° for 10 min. In the egg-albumin column the mean weights of the respective coagula are given. In this and following tables these conventions are used for digestion of caseinogen: 0 = no difference from blank; * = doubtful; + = partial digestion; ++ = complete digestion.

The organism *Proteus 3* was a stock culture from the London School of Hygiene[1] and thought to be Felix and Weil's *x* 19 strain. *Bacillus mesentericus* was a stock laboratory strain from the Pathological Laboratory, Cambridge[2]. *Pseudomonas 1* was isolated by the author from an abattoir but had been kept in culture for some months, and *Pseudomonas 2* was freshly isolated from cow-dung.

It will be seen that although sterile filtrates from all the above organisms liquefied gelatin and digested caseinogen beyond the acid-coagulable stage, filtrates from *Proteus 3* and *Pseudomonas 1* were without action on the native egg-albumin. Slight digestion beyond the heat-coagulable stage was apparently obtained with *B. mesentericus* and *Pseudomonas 2*. The latter organism was freshly isolated and sown immediately into broth, because it was thought that non-digestion of the albumin might be due to the fact that the stock strains had lost much of their vigour on repeated subculture. That this was not the case is evident from Table II in which it is seen that *Pseudomonas 2* produced a gelatinase so active that 0·01 cc. of filtrate liquefied 5 cc. of gelatin, while equal volumes of filtrate and albumin solution resulted in only the slight decomposition of albumin shown in Table I. Gelatin is apparently more easily attacked than caseinogen, since the higher dilutions which still liquefied gelatin were without action on the caseinogen.

[1] Supplied by courtesy of Dr Anderson.

[2] Supplied by courtesy of Dr Graham-Smith.

Table II. *Effect of dilution.*

Pseudomonas 2 grown for 8 days at 20°, filtrate incubated for 3 days at 37°.

Volume of filtrate added to 5 cc. gelatin or caseinogen cc.	Gelatin		Caseinogen	
	Blank	Exp.	Blank	Exp.
0·01	set 3 mins.	*not* set 24 hrs.	0	0
0·05	,, ,,	,, ,,	0	±
0·10	,, ,,	,, ,,	0	+
1·00	,, ,,	,, ,,	0	+ +
2·00	,, ,,	,, ,,	0	+ +

Apparently, therefore, little or no decomposition of pure native albumin beyond the heat-coagulable stage occurred after incubation with filtrates containing a very active gelatinase and a moderately active casease. Failure to decompose the albumin can hardly in this instance be explained on the basis of specificity of amino-acids in the food supply, for there are presumably present in meat-extract peptone broth all the amino-acids required. Several possibilities present themselves, but further experimental evidence with better methods is desirable. Since the main object of this paper was the study of protease formation in synthetic media, the point has not at present been pursued further.

Protease formation in synthetic media.

Methods.

In the main Merrill and Clark's simple medium [1928], the media of Diehl [1919] and Fränkel [1894], or slight modifications of them, have been used. The exact composition of these media as used is listed in the Appendix. Pyrex glassware was used throughout. It was cleaned carefully by standing for 24 hours under strong chromic acid, 24 hours under strong alkali, several days under distilled water repeatedly changed and was finally autoclaved at two atmospheres pressure for one hour. Ammonia-free redistilled water was used exclusively. All salts, being of A.R. standard to begin with, were recrystallised two or three times. Glycerol and lactic acid were distilled once *in vacuo*. Glucose was crystallised three times from a mixture of alcohol and water. Good samples of crystalline asparagine and glycine were recrystallised from water. In the case of Merrill and Clark's and Fränkel's media, stock solutions of each of the constituents were made up separately in sterile water and finally sterilised by steaming once for 15 minutes. The sterilisation of the mixture in the final medium, which invariably resulted in decomposition of the glucose, was thus avoided, the mixture being made up by dilution from stock solutions with aseptic precautions. In all cases the final p_H of the medium was adjusted prior to inoculation to 7·4 approximately by the colorimetric method, using $N/5$ NaOH stored in a waxed bottle fitted with delivery burette. The organisms were grown on the surface of agar plates at the appropriate temperature overnight. The surface growth was then carefully scraped off with a sterile platinum needle, taking care not to break into the agar, and suspended in saline. After

Organism		Clark with calcium and magnesium							Clark without calcium or magnesium						
		1	2	3	4	5	6	10	1	2	3	4	5	6	10
Proteus 1		+	*	0	.	.	.	.	+	0	.	.	.	.	.
Proteus 2		++	*	0	.	.	.	.	++	0	.	.	.	.	.
Proteus 3		+	*	0	.	.	.	.	+	0	.	.	.	.	.
"*Proteus 4*"		+++	+++	+++	+++	+++	+++	+++	++	+	+	+	*	.	.
B. mesentericus	20°	+++	+++	+++	+++	+++	+++	+++	+	0	.	.	.	.	.
	30°	+++	+++	+++	+++	+++	+++	+++	+	+	.	.	.	.	.
	37°	+++	+++	+++	+++	+++	+++	+++	++	+	*	0	.	.	.
Pseudomonas 1		+++	+++	+++	+++	+++	+++	+++	++	+	++	+	+	*.	.
Pseudomonas 2		+++	+++	+++	+++	+++	+++	+++	++	++	+	+	+	*	.
		Fränkel							Fränkel with calcium and magnesium						
Pseudomonas 1		+++	+++	+++	+++	+++	+++	+++	.	.	.	.	.	.	.
Pseudomonas 2		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
B. mesentericus	20°	+++	+++	+++	+++	+++	+++	+++	.	.	.	.	.	.	.
	30°	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	37°	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++

Convention used: + + + = Very heavily turbid. Luxuriant growth.

Numbers at the head o

Table III.

Medium																							
ium		Clark with calcium only						Clark with magnesium only							Diehl								
6	10	1	2	3	4	6	10	1	2	3	4	5	6	10	1	2	3	4	5	6	10		
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	0	.	..	.	.	.		
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	*	0	.	.	.	.		
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	*	0	.	.	.	.		
.	.	.	.	.	.	.	.	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +		
.	.	.	.	.	.	.	.	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	.	.	.	.	.	.	.		
.	.	+	+	+	*	.	.	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +		
.	.	.	.	.	.	.	.	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	.	.	.	.	.	.	.		
*.	.	+ +	+ +	+	+	*	.	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+	+ +	+ + +	+ +	+ + +	+ + +		
*	.	+ +	+ +	+ +	+ +	+	+	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +		
ium		Fränkel with calcium						Fränkel with magnesium															
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
+ +	+ + +	+ +	+ + +	+ +	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	.	.	.	.	.	.	.		
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		
+ +	+ + +	.	.	.	.	.	.	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	.	.	.	.	.	.	.		
+ +	+ + +	+ +	+ + +	+ +	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	.	.	.	.	.	.	.		

ant growth. + + = Heavily turbid. + = Definitely turbid. * = Doubtful. 0 = Negative, no growth.

at the head of the columns refer to the number of the subculture.

centrifuging at high speed for 30 minutes the deposit was ground up in a fresh quantity of sterile saline and the centrifuging repeated. In all, at least 3 washings were carried out. 1–2 cc. of the final suspension of about 50 cc. were inoculated into the various media. From 20 to 50 cc. of culture fluid were used in a thin layer on the bottom of 250–500 cc. Erlenmeyer flasks in order to ensure adequate aeration. Incubation was in general carried on from 2–5 days, for although the final growth was in many cases as luxuriant as in broth culture, the rate of growth was noticeably slower at the beginning. Doubtful cases were incubated for a week or more. At least ten subcultures were made to show that the organism was capable of continued growth in the synthetic medium. As a rule those organisms which could not flourish in the medium chosen died out at about the third subculture. Protease formation was measured in a similar manner to that used for broth cultures. Egg-albumin was dispensed with since the broth controls of the organism had given so little breakdown.

Results of experiments on growth in synthetic media.

The results of the experiments to determine the viability of the organisms in the respective media are summarised in Table III. *Proteus 1* was a strain of *P. vulgaris* obtained from the National Collection of Type Cultures, *Proteus 2* was isolated by the author from putrid ox-liver, *Proteus 3* was obtained as mentioned previously from the London School of Hygiene and was probably Felix and Weil's *x* 19 strain. "*Proteus 4*" was stated by one authority to be a true *Proteus*, by another to be a *Zopfius*.

It will be seen that the *Proteus* strains 1, 2 and 3 all consistently failed to grow in any of the synthetic media used. Slightly better growth was obtained in a medium containing the following unpurified amino-acids: glycine, alanine, asparagine, tryptophan, histidine, cysteine, cystine and tyrosine. Even here, however, growth failed after 4–5 subcultures. The passage of oxygen through the culture also had a stimulating influence but failed to maintain continued growth. "*Proteus 4*" (*Zopfius* ?) on the other hand grew well in Clark's and Diehl's media, provided salts of magnesium were present. In this connection it is interesting to note that Merrill and Clark [1928] reported good growth of "Proteus bacteria" in their synthetic media, whilst Wilson [1930] and Koser and Rettger [1919] obtained negative results in a variety of media. It is possible, but not yet proven, that certain strains of true *Proteus* organisms belong to the group which Hosoya and Kuroya [1923] showed would not grow without the presence of vitamin B. A broth control experiment indicated that protease formation with "*Proteus 4*" was feeble. Attempts to work with *Proteus* organisms had therefore to be abandoned. *B. mesentericus* and organisms of the *Pseudomonas* group proved suitable. Luxuriant growth was obtained in Merrill and Clark's simple medium with these bacteria provided salts of calcium and magnesium were present. Whilst requiring about 3 days to reach maximum turbidity as compared with 24 hours in broth, the final growth as

judged by turbidity was equally good in both cases. Growth in the absence of both calcium and magnesium was either feeble or definitely negative. In the presence of calcium but the absence of magnesium growth was little if any better than in the absence of calcium. In the presence of magnesium, on the other hand, luxuriant growth was again obtained. Good growth was also obtained in Diehl's medium which contained magnesium but no calcium. Apparently salts of magnesium are essential for the continued growth of bacteria in these simple synthetic media with an inorganic source of nitrogen. Such a salt is not universally necessary for growth, because continued multiplication of the bacteria took place in Fränkel's medium which contained no salt of calcium or magnesium. This medium, however, contains both ammonium lactate and asparagine. Even here these salts exerted a stimulating influence on growth, because, although good growth was obtained in their absence, more rapid and somewhat more luxuriant growth took place in their presence.

Protease formation.

The results of a number of experiments are summarised in Table IV. It will be seen that good protease formation took place in Merrill and Clark's medium in the presence of salts of calcium and magnesium, gelatin being liquefied and caseinogen digested. Little or no protease could be detected in the presence of calcium without magnesium in this medium, the growth, however, being relatively poor. Nevertheless counts showed that fair multiplication had occurred. In the same medium together with magnesium salts only luxuriant growth occurred in every way as good as in the presence of both salts. Yet in the latter case protease formation was negative or at the best very doubtful. Hence the position taken up by Wilson [1930] that these salts influence protease formation only in so far as they stimulate growth is not tenable. Either calcium salts alone, or a mixture of calcium and magnesium salts, have a specific action on the liberation of proteases in this medium. Magnesium salts alone have not this ability, or possess it only to a very slight extent, although they stimulate growth. The action of calcium salts only, or a mixture of calcium and magnesium salts, apart from their growth-stimulating properties, could not be tested in Merrill and Clark's medium, because so little growth occurred in their absence. A medium was required which would give good growth in the absence of these salts as well as in their presence. After some preliminary tests a slight modification of Fränkel's medium [1894] was adopted, as mentioned above. Continued growth of both *Pseudomonas* and *B. mesentericus* was obtained in this solution. All attempts to demonstrate the presence of protease in the medium without any addition of salts of calcium and magnesium have consistently failed, despite good growth with both organisms. A little enzyme was detectable in the presence of calcium salts, but good protease formation occurred on the addition of both calcium and magnesium salts. That the action of these salts was not due to activation of the enzyme during the incubation of a filtrate containing them and protein was shown by the fact that no difference

Table IV.

Temperature of incubation 37°.

Organism	Medium	Growth	Temp. of incubation of culture °C.	No. of sub-culture	Age of sub-culture days	Time of incubation of filtrate hrs.	Protease formation		
							Gelatin		
							Blank	Exp.	Caseinogen
Pseudomonas 2	Clark with Ca and Mg salts	+ + +	20	3	8	48	Set 3 mins.	>10 hrs.	+ +
,,	Clark without Ca and Mg salts	+	20	3	8	48	,, 3 ,,	3 mins.	0
,,	Clark with Ca salts only	+ +	20	3	7	48	,, 3 ,,	7 mins.	*
,,	,,	+ +	20	4	8	48	,, 3 ,,	6 mins.	*
,,	,,	+ +	20	5	4	48	,, 3 ,,	15 mins.	*
,,	Clark with Mg salts only	+ + +	20	3	7	48	,, 4 ,,	4 mins.	*
,,	,,	+ + +	20	4	8	48	,, 3 ,,	3 mins.	0
,,	,,	+ + +	20	5	4	48	,, 3 ,,	4 mins.	0
,,	Diehl	+ +	20	4	7	48	,, 4 ,,	5 mins.	0
,,	Fränkel alone	+ + +	20	5	9	48	,, 3 ,,	3 mins.	0
,,	,,	+ + +	20	8	4	48	,, 3 ,,	3 mins.	0
,,	Fränkel with Ca and Mg salts	+ + +	20	2	2	48	,, 3 ,,	>10 hrs.	+ +
,,	,,	+ + +	20	3	5	48	,, 3 ,,	>10 hrs.	+ +
,,	,,	+ + +	20	5	4	48	,, 4 ,,	>10 hrs.	+ +
,,	Fränkel with Ca salts only	+ + +	20	2	2	48	,, 3 ,,	3 mins.	*
,,	,,	+ + +	20	5	5	48	,, 3 ,,	4 mins.	0
,,	Fränkel with Mg salts only	+ + +	20	2	2	48	,, 3 ,,	3 mins.	0
,,	,,	+ + +	20	5	4	48	,, 3 ,,	4 mins.	*
B. mesentericus	Clark with Ca and Mg salts	+ + +	37	1	5	48	,, 4 ,,	>10 hrs.	+
,,	,,	+ + +	37	2	6	48	,, 4 ,,	>10 hrs.	+
,,	,,	+ + +	30	8	11	48	,, 3 ,,	>10 hrs.	+ +
,,	Clark with Ca salts only	+ +	30	3	11	48	,, 3 ,,	3 mins.	*
,,	Clark with Mg salts only	+ + +	30	3	11	48	,, 3 ,,	5 mins.	0
,,	,,	+ + +	37	1	5	48	,, 3 ,,	3 mins.	0
,,	Fränkel alone	+ + +	30	6	11	48	,, 3 ,,	3 mins.	*
,,	,,	+ + +	37	1	5	48	,, 3 ,,	4 mins.	0
,,	Fränkel with Ca and Mg salts	+ + +	37	3	6	48	,, 3 ,,	>10 hrs.	+
,,	Fränkel with Mg only	+ + +	37	3	6	48	,, 3 ,,	3 mins.	0

occurred on adding 0·2 cc. of their solutions to inactive filtrates together with protein, prior to incubation.

The original observation of Merrill and Clark is therefore confirmed. Salts of calcium and magnesium are apparently of fundamental importance to the bacterial cell for the liberation of proteases into the surrounding medium, in the media so far tested. The best effect is obtained by the combination of the two salts: magnesium salts alone have a stimulating influence on growth but affect protease production but little if at all. Calcium salts alone have no apparent stimulating influence on growth, but a trace of protease seems to be detectable in their presence. The action of these salts cannot be explained as a process of stimulation of growth only. So far, only ammonium chloride, ammonium phosphate, ammonium lactate and asparagine have been used as possible sources of nitrogen for the organism. All these can readily be obtained in a state of purity. It remains to be seen whether the action of these salts is of a fundamental nature and common to all media containing other and more complex sources of nitrogen. While there is as yet no experimental evidence, it is interesting to speculate that the action of salts of calcium and magnesium may in some way be linked up with their well-known characteristic effects at certain interfaces and their influence on the reversal of certain emulsions. It is possible that proteolytic enzymes are already present in the cell and assisting in the processes of metabolism, but that they are only secreted into the surrounding medium with a change in the cell surface caused by magnesium and calcium ions. In that case the calcium salts of serum may play an important rôle in the phenomena of immunity.

Summary.

1. Pure native egg-albumin is apparently more resistant to bacterial enzymes than commercial caseinogen or gelatin. Sterile filtrates of broth cultures of *B. mesentericus* and a *Pseudomonas* which readily attacked the latter had but little action on the albumin.

2. Mixtures of salts of calcium and magnesium, or magnesium salts alone, exert a stimulating influence on the growth of micro-organisms in simple synthetic media. Calcium salts alone have but little action in this respect.

3. Proteases are formed in simple synthetic media with ammonium chloride as the source of nitrogen provided that salts of calcium and magnesium are present. Little or no protease formation occurs in the presence of magnesium salts alone, despite good growth. In a medium containing ammonium lactate and asparagine protease formation is stimulated by these salts.

4. The influence of salts of calcium and magnesium on protease formation cannot be explained on the basis of stimulation of growth only.

The author wishes to express his thanks to Sir William B. Hardy who suggested the problem, to Dr G. S. Graham-Smith for advice and the supply of

cultures, to Dr C. G. Anderson for the supply of cultures, and to Mr J. Barlow for assistance in the experimental work.

REFERENCES.

Adair and Robinson (1930). *Biochem. J.* **24**, 993.
Bainbridge (1911). *J. Hyg.* **11**, 341.
Brunton and Macfadyen (1889). *Proc. Roy. Soc. Lond.* **46**, 542.
Diehl (1919). *J. Infect. Dis.* **24**, 347.
Fermi (1890). *Arch. Hyg.* **10**, 1.
Fränkel (1894). *Hyg. Rundschau.* **17**, 769.
Hosoya and Kuroya (1923). *Sci. Rep. Govt. Inst. Infect. Dis.* Tokyo, **2**, 233, 287.
Koser and Rettger (1919). *J. Infect. Dis.* **24**, 301.
Merrill and Clark (1928). *J. Bact.* **15**, 267.
Sperry and Rettger (1915). *J. Biol. Chem.* **20**, 445.
Wilson (1930). *J. Bact.* **20**, 41.

APPENDIX. *Composition of media used.*

Merrill and Clark's medium.

NH_4Cl	10 g.
$Na_2HPO_4, 2H_2O$	10 g.
KH_2PO_4	2 g.
Glucose	10 g.
Water	1 litre

5 cc. of 0·1 % $MgSO_4, 7H_2O$ and 2 cc. well shaken suspension of 0·1 % $CaCO_3$ in 50 cc. of medium where required. Final p_H 7·4.

Diehl's medium.

$(NH_4)_2HPO_4$	1·75 g.	
K_2HPO_4	0·25 g.	
$Na_2HPO_4, 2H_2O$	0·5 g.	Final p_H adjusted to 7·4
$MgSO_4, 7H_2O$	0·5 g.	
Glycerol	20·0 cc.	
Water	1 litre	

Fränkel's medium.

NaCl	5·0 g.	
KH_2PO_4	2·0 g.	Final p_H adjusted to 7·4
Ammonium lactate ...	6·0 g.	
Asparagine	4·0 g.	
Water	1 litre	

Calcium and magnesium salts added as above when required.

CC. THE EFFECT OF HYDROGEN ION CONCENTRATION ON THE PRECIPITATING AND PROTECTIVE ACTIONS OF MIXTURES OF TWO PROTEINS ON COLLOIDAL GOLD AND GUM BENZOIN.

By REGINALD HAYDN HOPKINS, WILLIAM OGILVY KERMACK and WALTER THOMAS SPRAGG.

From the Biochemical Laboratory, Heriot-Watt College, and the Research Laboratory of the Royal College of Physicians, Edinburgh.

(*Received September 23rd, 1931.*)

In a recent communication [Hopkins, Kermack and Spragg, 1931] the results were given of a series of experiments on the precipitation of colloidal gold and gum benzoin by the proteins, gelatin, ovalbumin, serum-albumin, the serum-globulins and oxyhaemoglobin at different concentrations of protein and of hydrogen ions. Observations were also made on the protective actions of these proteins against precipitation of the colloids by 1·25 % sodium chloride. Except in the case of oxyhaemoglobin, the protective power was minimal in the neighbourhood of the isoelectric point of the protein. It seemed desirable to extend the observations to mixtures of two proteins in order to find out, for example, what occurs when conditions are such that one protein alone will precipitate and the other protect. It was decided for this purpose to limit the experiments to the three proteins, gelatin, ovalbumin and oxyhaemoglobin. Of these three, the first two have approximately the same isoelectric point, p_H 4·7, whilst that of the third is considerably higher, p_H 6·8. Further, gelatin has considerable protective power even in the neighbourhood of the isoelectric point, but ovalbumin has not, whilst the behaviour of oxyhaemoglobin is anomalous in that the minimum of protective action is at a p_H somewhat below that of the isoelectric point. These three proteins have been employed in pairs, and in each case two sets of observations have been made, colloidal gum benzoin being employed as lyophobic colloid in the one case and colloidal gold in the other. As in the previous experiments, it was desired to make readings over a range of p_H values. With each pair of proteins, sets of observations were made in which the p_H was kept constant but the concentration of each protein independently varied. Similar sets of observations were made at other p_H values. With each pair of proteins, either two or three sets of readings were made, each at a constant p_H. The values of p_H employed in the various cases were chosen so as to be likely to yield the maximum amount of information.

In all cases readings were made before and after the addition of sodium chloride.

Although mixtures of proteins are frequently encountered in practice, there appears to be comparatively little recorded in the literature in respect of the precipitating and protective actions of such mixtures. Reference may be made to a paper by Prideaux and Howitt [1931] on the rate of electrophoresis of gold sol in the presence of ovalbumin, gelatin and mixtures of the two. On the acid side of the isoelectric point, the rate of electrophoresis in the presence of the two proteins was considerably less than in the presence of either separately, whilst on the alkaline side, the rate was approximately the same as that observed in the presence of gelatin alone.

Experimental.

The general method of carrying out the experiments was analogous to that employed in the previous work [1931]. To each of a series of tubes were added 0·25 cc. of protein A and 0·25 cc. of protein B, each of suitable concentration, 0·25 cc. of a buffer solution and 1·0 cc. of the lyophobic colloid. The buffer solution contained $N/50$ phosphate and $N/50$ acetate. In any particular experiment the tubes were arranged in the form of a rectangular array. The tubes in any one row contained the same concentration of protein A but various concentrations of protein B, whilst the tubes in any one column contained the same concentration of protein B but various concentrations of protein A. The same buffer mixture was added to all the tubes in any one array, so that the final contents of all the tubes were approximately at the same p_H. This was ensured by previously adjusting the p_H of the protein solutions distributed to the tubes to be approximately that of the buffer mixture employed. To each tube of the array was added 1·0 cc. of either colloidal gum benzoin or of colloidal gold. The tubes were shaken and readings were made after 24 hours. 0·25 cc. of 10 % sodium chloride was then added to each tube, the tubes shaken again and read after a further 24 hours. The p_H of a number of tubes was then determined in order to ascertain the exact p_H, which, although approximately constant throughout the array was not necessarily the same as that of the buffer used, as the latter may be modified by the buffering substances present in the gum benzoin and the gold. As mentioned in the previous paper, the readings in the case of tubes containing appreciable quantities of oxyhaemoglobin could best be made by examining the contents in a beam from an arc lamp, when the presence of particles of gold throughout the solution could readily be detected by means of the Tyndall effect. In recording the results obtained with the gelatin-ovalbumin mixtures and gold sol, two observations were made for each tube, one referring to the colour and the other to the amount of gold which had settled to the bottom of the tube. In the case of experiments with gold in which oxyhaemoglobin was present, analogous observations of colour could only be made when the concentration of oxyhaemoglobin was small. In the previous paper these two methods of reading were not

clearly distinguished. When gold is precipitated by an inorganic salt the colour is changed from red to blue and precipitation follows. This precipitate is always quite blue. In the previous paper it was noted that in the presence of protein the precipitate was frequently red or purple, although all the gold had been deposited. The diagrams given showed the regions in which complete or almost complete colour change, or complete or almost complete precipitation, had been observed. It has become clear during the present work that it is desirable to distinguish between these two phenomena. As a consequence readings have been made in the case of gold, firstly on the basis of colour change alone, and secondly on the basis of degree of precipitation alone. The diagrams which are given have been prepared from the latter readings. It has been found that the two sets of readings did in fact follow each other fairly closely with regard to the general shape of the zones, but not with regard to the exact position of the zones. It also appears from the present results that the precipitates marked red or purple (by shading) in the previous paper, although distinctly reddish as compared with the normal blue precipitate of gold, were nevertheless considerably bluer than the unprecipitated gold. It follows from these considerations that the exact limits of precipitation of gold recorded in the new diagrams are not always completely in agreement with those given in the previous paper. This refers more particularly to the case of gelatin and gold. Fig. 8 shows the results when the new method of recording is employed. (Contrast with Fig. 1 A in the previous paper.) It will be noted that on the acid side of the isoelectric point, protection now occurs in the presence of lower concentrations of gelatin. This is because a tube showing an almost complete colour change is now in the non-precipitated region, as under these conditions with very low protein concentration, precipitation does not occur in general until the colour change is complete.

The experimental results are shown in Figs .1 A to 8. The thick lines, whether continuous or dotted, represent approximately the concentrations at which partial precipitation occurs, so that they separate zones of precipitation from those of non-precipitation. The shading indicates that side of the line on which is the zone of precipitation. Each line is distinguished by a number, *e.g.* 5·6, which denotes the p_H value to which the line refers. The significance of dotted and continuous lines, thick and thin lines, will be explained in the discussion.

Discussion.

According to the theory of colloids which has been elaborated in recent years by Kruyt and others, the stability depends on two factors, (1) the existence of an electric charge on the particles, and (2) the existence of groups which have an affinity for the medium employed for the continuous phase. In the case of a lyophobic colloid protected by a lyophilic colloid such as protein, the effects of the protein may be exerted in two different ways, (1) by modifying the electric charge on the particles, and (2) by forming a film round the

particles, so modifying the surface forces that the particles will not adhere together even though electrically neutral. In this paper, we propose to refer to the former as the "electrostatic effect" and to the latter as the "proper protective effect." For example, the "proper protective effect" of ovalbumin is relatively small, and that of gelatin relatively great. It has been shown in the previous paper [Hopkins, Kermack and Spragg, 1931] that when a single protein is present these two factors explain completely the behaviour of certain lyophobic colloids in the presence of proteins, although, with other lyophobic colloids such as gold, the behaviour is less simple. The results of the present work indicate that this conclusion, so far as concerns colloidal gum benzoin, may be extended to the case where two proteins are present simultaneously. It is entirely in harmony with the above principles that the proper protective effect should be additive and should always exert itself whatever the electrostatic condition of the particles may be. The electrostatic effect is also additive in itself, but is subordinate to the combined proper protective effect.

Precipitation of colloidal gum benzoin. We shall first consider the pair of proteins, ovalbumin and gelatin. In the previous paper, dealing with single proteins, it was found convenient to represent the results diagrammatically in the form of graphs, one ordinate representing concentration of protein, the other p_H. The regions over which complete or almost complete precipitation occurred were then marked out. As, in the present instance, there are two proteins, a three-dimensional model is required to give a similar representation. It was found possible from the observations to construct a model of this kind showing the regions precipitated, the three variables being concentration of gelatin, concentration of ovalbumin and p_H. It should be added that, in constructing this model, considerable help was gained by taking into account the degree of precipitation in tubes which were not exactly at the boundary line between precipitation and non-precipitation. It is difficult to give an adequate

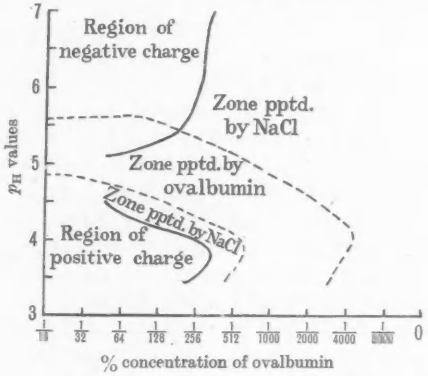


Fig 1 A. Action of ovalbumin on gum benzoin. Final concentration of buffers used $N/400$ acetate + $N/400$ phosphate. Isoelectric point of ovalbumin p_H 4·6–4·8.

representation of a model of this kind by two-dimensional diagrams, but some indication of its general nature may possibly be afforded by the "contour" diagrams given. Considering for the moment Figs. 1 A and 1 B, these refer to

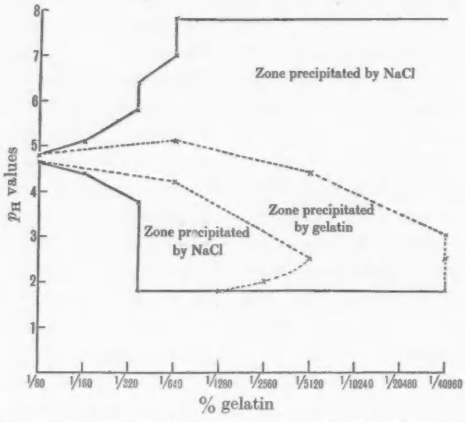


Fig. 1 B. Action of gelatin on gum benzoin. No buffers used.

ovalbumin and gelatin respectively alone, and so give a picture of the two adjacent faces of the model, which meet in an edge representing the p_H axis corresponding to zero concentration of both proteins. The region of precipitation in the three-dimensional model was in the form of a zone separating a region in which the particles possessed a positive charge from one in which they possessed a negative charge. This zone naturally had an under surface bounding the region of positive charge and an upper surface bounding the region of negative charge. The lines shown in Fig. 2 A may be regarded as contours drawn on these surfaces, each at a constant p_H. The parts of the contours which refer to the upper surface are represented by continuous lines and those referring to the lower surface by dotted lines. The thick continuous and dotted lines were obtained directly from the observations as mentioned in the experimental section. The thin continuous and dotted lines have been interpolated with the help of the model, and serve to indicate more fully the nature of the two surfaces, upper and lower. As mentioned in the experimental section, shading indicates on which side of the contours precipitation occurred.

The zone of precipitation comprises the region in which the particles would be expected to have a very low or zero charge, but it does not extend to any concentration of gelatin above a certain critical concentration. At this concentration the proper protective effect begins to function, so that, even at the isoelectric point, precipitation does not occur. Although the proper protective effect of the ovalbumin is not sufficient to prevent agglutination of the particles when their charge is zero, nevertheless it is not altogether non-existent. This is shown by the fact that, in presence of large concentrations of ovalbumin the

concentration of gelatin required for complete protection is not quite so great as when ovalbumin is absent (see Fig. 2 A).

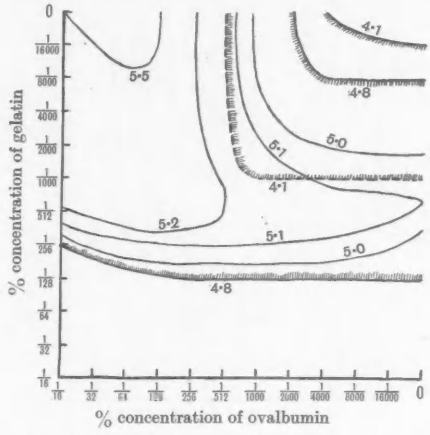


Fig. 2 A. Action of mixtures of gelatin and ovalbumin on gum benzoin at various [H·].

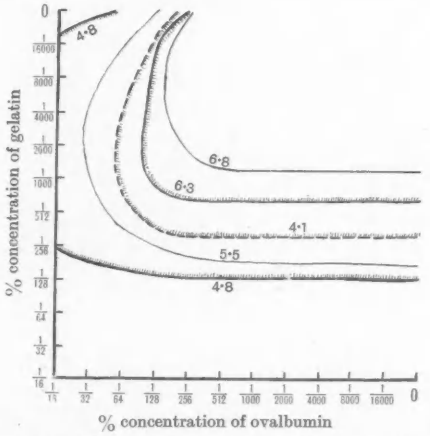


Fig. 2 B. Protective action of mixtures of gelatin and ovalbumin on gum benzoin at various [H·] in presence of 1·25 % NaCl.

In the case of mixtures of gelatin and oxyhaemoglobin, the same general result is obtained, and in the three-dimensional model the chief difference as compared with the gelatin-ovalbumin system is that the zone of precipitation is moved to a region of higher p_H values in that part of the system which refers to low gelatin and high oxyhaemoglobin concentrations (see Fig. 3 A).

In the case of ovalbumin-oxyhaemoglobin mixtures, the zone of precipitation is extended in the direction of higher ovalbumin concentration in consequence of the low proper protective effect of this protein. Since the proper

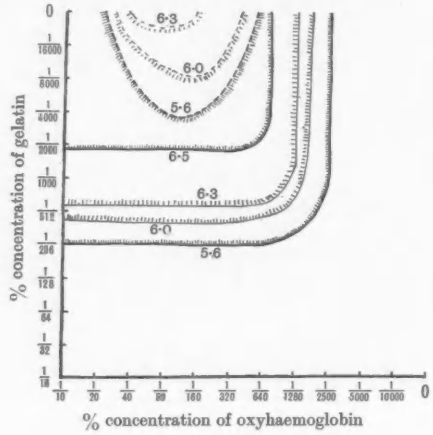


Fig. 3A. Action of mixtures of gelatin and oxyhaemoglobin on gum benzoin at various [H·].

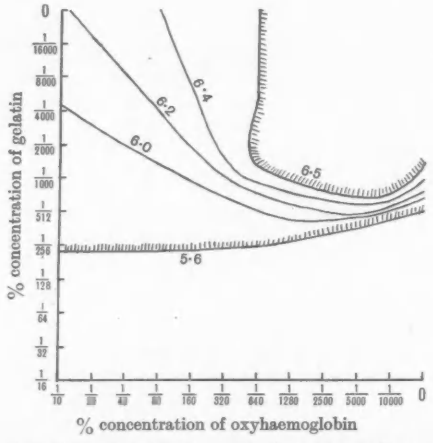


Fig. 3B. Protective action of mixtures of gelatin and oxyhaemoglobin on gum benzoin at various [H·] in presence of 1·25 % NaCl.

protective effects of these two proteins are small, the stability is almost entirely controlled by the electrostatic effects (see Fig. 4A).

Protection of colloidal gum benzoin. The results observed in the presence of sodium chloride may be discussed in the light of the same principles. The chief

function of salt is to reduce the charge on the particles which have no film of protein round them, or which have adsorbed only small quantities of protein.

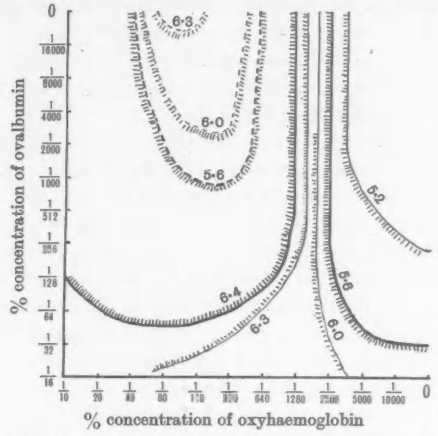


Fig. 4A. Action of mixtures of ovalbumin and oxyhaemoglobin on gum benzoin at various [H·].

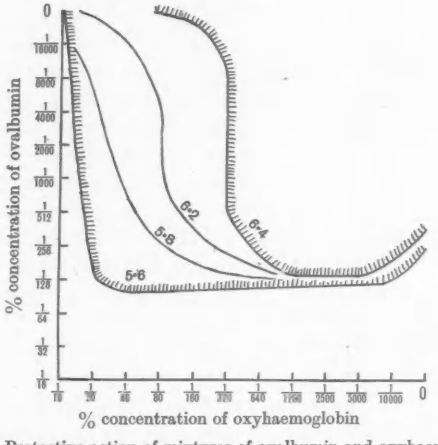


Fig. 4B. Protective action of mixtures of ovalbumin and oxyhaemoglobin on gum benzoin at various [H·] in presence of 1·25 % NaCl.

The salt may, however, also affect both the electrostatic and the proper protective effects of the protein. As shown in the previous paper, the minimum protective actions of ovalbumin and gelatin are in the neighbourhood of the isoelectric point, whilst that of oxyhaemoglobin is at about p_H 6, somewhat below the isoelectric point. Mixtures of ovalbumin and gelatin behave in

additive fashion, the protective effect of each accentuating that of the other, with one exception. When very small quantities of gelatin are present, very slightly more ovalbumin is required to protect than when no gelatin is present. This may be due to the effect of a very small quantity of gelatin in the surface layer of the particles, quite inadequate in itself to produce any appreciable protective action, so altering the surface forces that the quantity of ovalbumin adsorbed becomes decreased (see Fig. 2B).

Systems of oxyhaemoglobin and gelatin, or of oxyhaemoglobin and ovalbumin also in general behave additively. The results obtained with these two systems are very similar to each other (see Figs. 3B and 4B). This similarity is particularly marked, as, in the presence of salt, high concentrations of ovalbumin exercise a protective action, even at the isoelectric point, although the minimum at this point is rather more marked than in the case of gelatin. Since, in the absence of salt, ovalbumin precipitates at the isoelectric point even in high concentrations, this result implies a certain amount of peptisation by the sodium chloride, similar to that previously noted in respect to oxyhaemoglobin at p_H 6·5. In the previous paper, the observations on ovalbumin in the absence of salt were not recorded, so that peptisation was not noted. The most satisfactory explanation of the anomalous behaviour of oxyhaemoglobin in the presence of salt, as recorded in the previous paper and confirmed in this one, is that the proper protective effect is minimal not at the true isoelectric point, but at about one unit of p_H below. This shift in the minimum of proper protective effect may or may not be accompanied by a corresponding change in the apparent isoelectric point in the presence of sodium chloride, similar to that noted by Michaelis and Szent-Györgyi [1920] in the case of caseinogen.

The general results in the two systems, oxyhaemoglobin-gelatin and oxyhaemoglobin-ovalbumin, are in harmony with the principles enunciated above, namely, that the proteins independently affect the electric charge, and also independently and additively exercise their proper protective effects. In accordance with the curious effect of salt on the protective action of ovalbumin, it is found that the similar peptising action of salt on oxyhaemoglobin is not inhibited by the presence of ovalbumin, although it seems to be inhibited by the presence of moderate quantities of gelatin.

Precipitation and protection of colloidal gold. Anomalous effects of the type referred to above in connection with oxyhaemoglobin and gum benzoin were more marked when this protein was used with gold. This was referred to in the previous paper. The further complication arises here, that, as mentioned in the experimental section of this paper, the degree of colour change and the extent of precipitation do not necessarily run parallel in the presence of protein. When the precipitation results for two proteins *plus* gold are examined, they are found broadly to conform with the principles enunciated above in the case of gum benzoin, with the modification necessitated by the existence of the anomalies exhibited in the case of oxyhaemoglobin. When the colour change results are examined, they are also found to be in accordance with the above

principles. The lack of parallelism between colour change and precipitation is most marked in the presence of gelatin on the acid side of the isoelectric point, where considerable colour change without flocculation takes place, whilst in

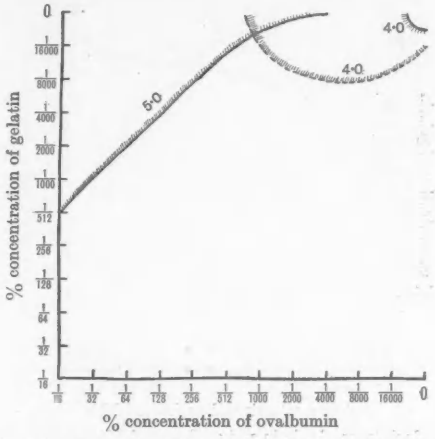


Fig. 5A. Action of mixtures of gelatin and ovalbumin on gold sol at various [H·].

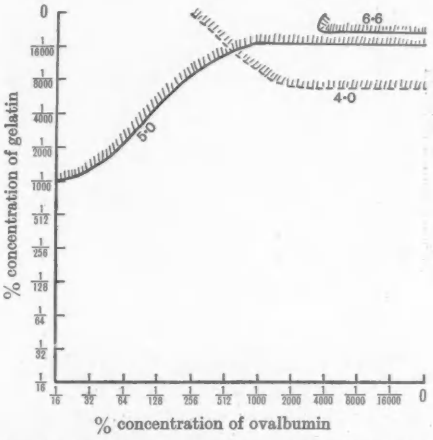


Fig. 5B. Protective action of mixtures of gelatin and ovalbumin on gold sol at various [H·] in presence of 1·25 % NaCl.

the neighbourhood of the isoelectric point of the protein, complete flocculation is observed without equally complete colour change. These remarks are true of mixtures both in the presence and absence of salt.

It was suggested that the probable explanation lay in the small adsorption

of the protein on the gold, and the poor protective action of these proteins on the acid side as compared with that on the alkaline side of the isoelectric point suggests that this small adsorption may be characteristic of the protein cations.

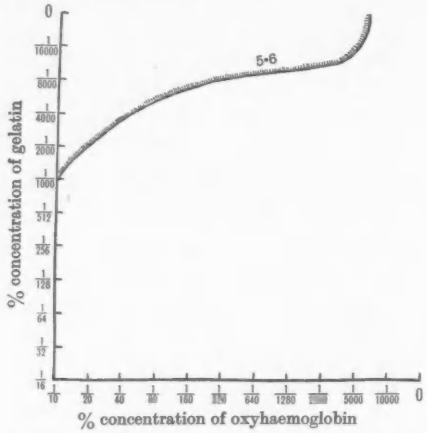


Fig. 6A. Action of mixtures of gelatin and oxyhaemoglobin on gold sol at various [H·

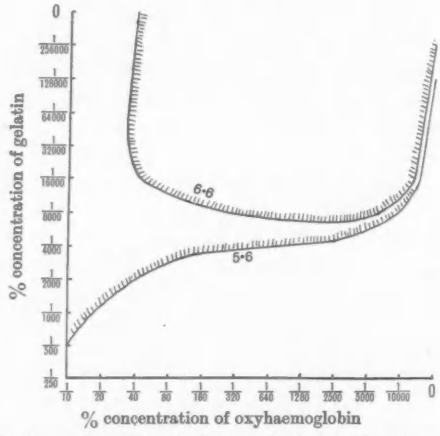


Fig. 6B. Protective action of mixtures of gelatin and oxyhaemoglobin on gold sol at various [H·] in presence of 1·25 % NaCl.

The proper protective effect of the proteins will be measured by the quantity required to prevent precipitation when the gold particles are discharged and the protein molecules are in the neutral condition. The much greater protective action when the p_H is somewhat greater or less than corresponds to the isoelectric point must be regarded as the combined effect of the proper protective

effect of the protein along with stabilisation due to the existence of like charges. On this basis we may consider for example the results obtained with oxyhaemoglobin and gelatin at p_H 5·6 (see Figs. 6 A and 6 B). In the absence of salt,

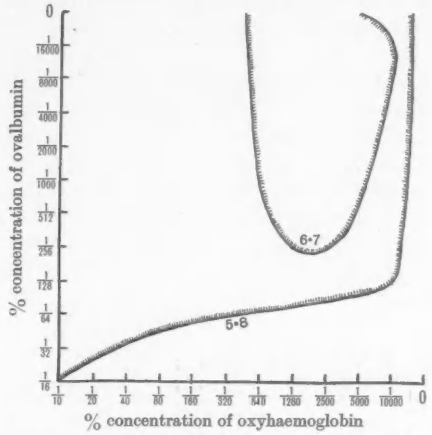


Fig. 7 A. Action of mixtures of ovalbumin and oxyhaemoglobin on gold sol at various [H·].

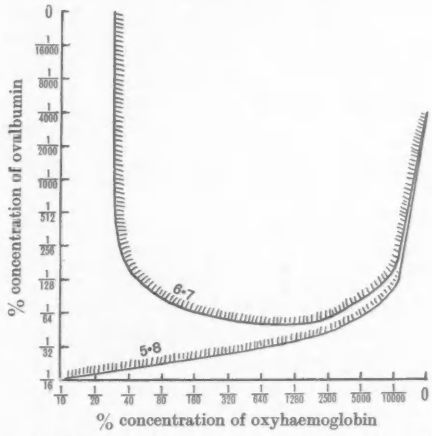


Fig. 7 B. Protective action of mixtures of ovalbumin and oxyhaemoglobin on gold sol at various [H·] in presence of 1·25 % NaCl.

there is no precipitation with gelatin alone. In the presence of a sufficient concentration of oxyhaemoglobin, however, precipitation occurs with zero or very small concentrations of gelatin. But with larger concentrations of gelatin the negatively charged gelatin ions reinforce the negative charge on the particles and in this way prevent discharge by the positively charged oxyhaemoglobin.

As the concentration of oxyhaemoglobin increases up to a point, rather more gelatin is required to prevent precipitation. In the presence of salt and absence of oxyhaemoglobin gelatin protects the gold at a concentration of 1/128,000 % and over. At a somewhat lower p_H gelatin does not protect at 1/8000 % concentration. It seems probable that the protective action of gelatin at p_H 5·6 is accentuated by the negative charge borne by the protein at this p_H. When, however, small quantities of oxyhaemoglobin are present, the positive charge on this protein will tend to neutralise the negative charge on the gelatin. The protection of the colloid will then have to be effected largely by the proper

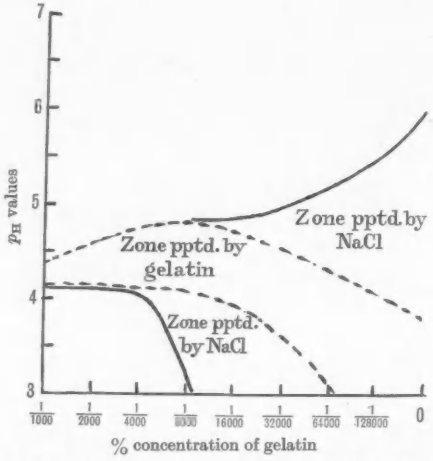


Fig. 8. Action of gelatin on gold sol. Final concentration of buffers used $N/400$ acetate + $N/400$ phosphate. Isoelectric point of gelatin p_H 4·7.

protective effect of the gelatin assisted by the comparatively small proper protective effect of the oxyhaemoglobin, the latter on the acid side of the isoelectric point. Thus, protection does not take place in the presence of 1/10,000 % oxyhaemoglobin until the concentration of gelatin exceeds 1/8000 %, and this quantity of gelatin increases as the concentration of oxyhaemoglobin increases. The concentration of gelatin never exceeds 1/500 % even when the concentration of oxyhaemoglobin is as high as 1/10 %. The limiting factor here may be the proper protective effect of the gelatin. The results obtained with the other mixtures of proteins conform to the same principles. In the case of gelatin and ovalbumin the results obtained are similar to those obtained with this mixture of proteins and gum benzoin (Figs. 5 A and 5 B) whilst those obtained with ovalbumin and oxyhaemoglobin are similar to those obtained with gelatin and oxyhaemoglobin with gold (Figs. 7 A and 7 B).

SUMMARY.

1. Experiments have been performed on the precipitation of colloidal gold and gum benzoin by mixtures of ovalbumin and gelatin, ovalbumin and oxyhaemoglobin, and of oxyhaemoglobin and gelatin at various hydrogen ion concentrations.

2. Observations have also been made on the protective action of these mixtures at various hydrogen ion concentrations against precipitation of the lyophobic colloids by 1·25 % sodium chloride.

3. It appears that the results previously obtained with the single proteins can be extended to apply to the above mixtures. If the effect of a protein on the charge of the lyophobic particles is distinguished from its proper protective effect, the two proteins would appear to exercise each of these two influences additively.

REFERENCES.

Hopkins, Kermack and Spragg (1931). *Biochem. J.* **25**, 829.
Michaelis and Szent-Györgyi (1920). *Biochem. Z.* **103**, 178.
Prideaux and Howitt (1931). *Biochem. J.* **25**, 391.

CCI. INFLUENCE OF PROTEINS AND PROTEIN DERIVATIVES ON THE ENZYMIC HYDROLYSIS OF STARCH BY MALT DIASTASE.

By BRONISŁAW FILIPOWICZ.

From the Biochemical Laboratory, Faculty of Medicine, the University, Warsaw.

(*Received September 28th, 1931.*)

Much of the work published by earlier authors on the activation or inactivation of amylase by various substances, such as amino-acids, amines, amides, proteins, *etc.*, is vitiated by the fact that possible variations in the p_H of the medium due to the introduction of the substances examined were not taken into consideration. The same applies to other factors influencing the activity of this enzyme, such as purity, temperature, *etc.*

Euler and Josephson [1926] found that the addition of glucose or laevulose retarded the velocity of enzymic amylolysis whilst addition of amines or amino-acids to such systems accelerated the reaction. Sherman, Caldwell, and Adams [1926] considered that the activating effect of amino-acids was due to their inhibiting action on the autolysis of the enzyme. Przyłęcki and Majmin [1931] have shown that proteins can combine with, and consequently possibly activate the substrate, the combination between starch and proteins being dependent on the p_H of the medium. It follows that amylolysis in presence of protein should depend quantitatively on the p_H of the medium, and in the present paper work on this aspect of the problem will be described.

Experimental.

Methods.

Solutions of Merck's amylase (Diastase absolut) were prepared separately for each series of determinations by dissolving 0·1 g. of amylase in 100 cc. of 0·1 % NaCl, and filtering after 30 min. Solutions of Merck's soluble starch or dextrin were prepared by gradual addition of starch or dextrin paste to boiling water, boiling a further 15 min., and, after cooling, diluting to the requisite volume. Two buffer solutions were used: Walpole's acetate mixtures below p_H 4·5, and Sørensen's phosphates above p_H 4·5. p_H was determined colorimetrically in some cases but chiefly potentiometrically. Reducing sugars obtained from starch were determined by Bertrand's method, using 60 cc. of solution and 20 cc. each of Bertrand's reagents. Merck's ovalbumin (6 g.) was dissolved in 100 cc. of water, and filtered after 30 min.; the filtrate had

p_H 7·27. The solutions were freed from protein before determination of sugar by addition of 30 cc. of a mixture of 200 cc. of 4·5 % $ZnSO_4$ and 40 cc. of 0·1 *N* NaOH, heating for 3 min. at 100°, and filtering. 60 cc. of the filtrate were taken for determination of sugar. Peptone solutions (Poulenc) were prepared in an analogous manner; their p_H was 4·83. Gelatin (Coignet Père et Fils) was dissolved in water at 90°; the solutions had p_H 5·89.

A. *Enzymic hydrolysis of starch at varying* p_H.

To a number of 200 cc. flasks 15 cc. of 3 % starch, 15 cc. of the appropriate buffer solutions, 1 cc. of 0·1 % amylase and 29 cc. of water were added. The flasks were then left for 1 hour at 30°, and maltose was determined. The results (Table I) indicate that the optimal p_H is, under the given conditions, 4–4·5. In more acid solutions the activity of amylase rapidly falls, being practically zero at p_H 3·0.

Table I. *Enzymic hydrolysis of starch at varying* p_H*.

p_H	mg. maltose	p_H	mg. maltose
3·0	5·1	6·0	59·5
3·5	57·4	6·5	54·1
4·0	65·8	7·0	48·2
4·5	64·0	7·5	37·5
5·0	62·2	8·0	25·3
5·5	59·8	—	—

* Results in all Tables are the mean of 4 observations.

B. *Action of amylase in the presence of ethyl alcohol.*

1. *Velocity of reaction in various concentrations of alcohol.* A series of flasks was prepared containing 30 cc. of 1·5 % starch, 10 cc. of buffer solutions p_H 5·6, 18 cc. of aqueous alcohol, so as to obtain concentrations of 1, 5, 10, 15 and 20 % of alcohol in the whole system, and 2 cc. of 0·1 % amylase. The systems containing 15 and 20 % of alcohol were turbid, owing to partial precipitation of starch. All flasks were kept 2 hours at 30°. The results (Table II) show that percentage retardation of reaction is directly proportional to the concentration of alcohol.

Table II. *Influence of conc. of alcohol on amylolysis at* p_H *5·6.*

% alcohol	mg. maltose	% retardation
0	89·6	—
1	87·5	2·3
5	83·5	6·8
10	78·3	12·6
15	71·2	20·5
20	64·6	27·9

2. *Velocity of reaction in 5 % alcohol at varying* p_H. A number of flasks were prepared, containing 15 cc. of 3 % starch, 15 cc. of buffer solutions, 3·125 cc. of alcohol, 25·875 cc. of water, and 1 cc. of 0·1 % amylase. The systems were left 1 hour at 30°. The results (Table III) are similar to those

obtained in the absence of alcohol, maximum reaction taking place at p_H 4·5. Reaction is retarded at p_H above 4·5 by alcohol to an extent increasing with p_H.

Table III. *Amylolysis in 5 % alcohol at varying* p_H.

	mg. maltose		
p_H	With alcohol	Without alcohol	% retardation due to alcohol
3·5	51·5	52·8	2·6
4·5	58·4	60·6	3·7
6·0	48·5	55·3	12·5
7·5	23·2	32·3	28·7

3. *Reaction in alcoholic media with varying concentrations of starch.* Two series of flasks were prepared, one containing 0·5 and the other 1·5 % starch; in both series alcohol was present in concentrations of 0, 1, 5, 10, 15 and 20 %. The systems, consisting of 30 cc. of starch, 10 cc. of buffer solution (p_H 6·5), 19 cc. of aqueous alcohol or water, and 1 cc. of 0·1 % amylase, were kept at 30° for 2 hours. The results (Table IV) indicate that percentage retardation of reaction due to alcohol is practically independent of the concentration of starch taken. At the same time the yield of maltose is about 30 % greater using 1·5 than 0·5 % starch, both in the presence and absence of alcohol.

Table IV. *Amylolysis at various concentrations of alcohol and starch.*

	0·5 % starch		1·5 % starch	
% alcohol	mg. maltose	% retardation	mg. maltose	% retardation
0	41·0	—	54·7	—
1	39·5	3·6	53·8	1·6
5	36·2	11·7	51·1	6·5
10	34·7	15·3	47·1	13·9
15	31·9	22·2	41·7	23·7
20	28·7	30·0	35·4	35·2

4. *Comparison with dextrin.* The retarding action of alcohol may be due to partial precipitation of starch. Should this be the case, the retarding action of alcohol should be absent from systems containing dextrin, which is not precipitated by the concentration of alcohol used. The following experiment shows that this is not the case. A number of flasks each containing 30 cc. of 1·5 % starch or dextrin, 10 cc. of buffer solution (p_H 6·5), 12·5 cc. of 96 % alcohol or water, 5·5 cc. of water and 2 cc. of 0·1 % amylase were left 2 hours at 30°, after which maltose was determined. Since the dextrin used contained some reducing sugars, the value found for systems such as the above to which 2 cc. of water had been added in place of amylase, was deducted from the reducing sugar content of the above systems. The results obtained were substantially the same for starch as for dextrin; thus 86·5 mg. of maltose were produced from starch in the absence of alcohol and 62·9 mg. in its presence, a retardation of 26·8 %. The corresponding values for dextrin are 37·0 and 27·6 mg., and the retardation is 24·3 %.

5. *Action of alcohol on amylase.* 0·1 g. of amylase and 0·1 g. of NaCl were dissolved in 100 cc. of 20 % alcohol, and kept 1 hour at 30°, together with a

similar aqueous solution. A number of systems, containing 30 cc. of 1·5 % starch, 10 cc. of buffer solution (p_H 6·5), 12·5 cc. of alcohol or water, 2·5 cc. of water, and 5 cc. of aqueous or 20 % alcoholic amylase solution, were kept 1 hour at 30°, and reducing sugar was determined. The activity of amylase previously subjected to the action of alcohol was smaller than was that of amylase dissolved in water, in both alcoholic and aqueous solutions (81·5 and 84·7 mg. of maltose produced respectively, as compared with 109 and 111·5 mg. in the presence of aqueous amylase).

Four solutions of amylase were now prepared, as follows:

Amylase I. 0·4 g. of amylase and 0·4 g. of NaCl in 100 cc. of 20 % alcohol;

Amylase II. The same, in 5 % alcohol;

Amylase III. The same, in 1 % alcohol;

Amylase IV. The same, in water.

These 4 solutions were kept for 22 hours at 30°. Solutions I and II remained clear, whilst solutions III and IV contained a flocculent precipitate. Four series of flasks were prepared, each containing 30 cc. of 1·5 % starch, 10 cc. of buffer solution (p_H 6·5), 19·5 cc. of water, and 0·5 cc. of amylase solution I, II, III, or IV. The maltose produced after 1 hour at 30° was 89·5 mg. by amylase I, 85 mg. by amylase II, 88·3 by amylase III, and 74·6 mg. by amylase IV. Alcohol therefore inhibits the inactivation of amylase in solution.

Three volumes of water were now added to amylase I, II and IV, yielding amylase I′ (0·1 %, in 5 % alcohol), II′ (0·1 %, in 1·25 % alcohol), and IV′ (0·1 % in water). These solutions were kept 2 hours at 30°, and 2 cc. of each were added to similar systems as in the preceding experiment. Maltose determined after 2 hours at 30°, amounted to 74·6 mg. with amylase I′, 66·6 mg. with amylase II′, and 59·2 mg. with amylase IV′.

The same experiment was repeated with amylase solutions I′, II′ and IV′, kept a further 24 hours at 30° before addition to the systems, the maltose after 3 hours at 30° now being respectively 102, 68·1, and 50·1 mg.

It appears that the protective influence of alcohol on amylase becomes accentuated with duration of contact, the relative acceleration of action of amylase in 5 % alcohol amounting after 22 hours to 15 %, after 24 hours to 20 %, and after 48 hours to 100 %.

C. *Action of amylase in the presence of proteins and of their derivatives.*

1. *Influence of ovalbumin at varying* p_H. The action of amylase was examined in freshly prepared systems containing ovalbumin buffered to p_H 3·5, 4·5, 6 and 7·5. A number of 200 cc. flasks were prepared, containing 15 cc. each of 3 % starch, buffer solution, and 6 % ovalbumin solution or water, 14 cc. of water and 1 cc. of 0·1 % amylase. All systems were kept 1 hour at 30°, after which 20 cc. of Bertrand's solution I were added to the control flasks in order to interrupt the reaction, whilst 30 cc. of Hagedorn-Jensen solution

were added to the flasks containing albumin, which was then coagulated by the method described above. The systems were filtered, and 60 cc. of the filtrates were taken for determination. Maltose was determined by Bertrand's method, and the result was multiplied by 1·5 in order to obtain the value for the whole system. The results (Table V) indicate that the action of ovalbumin is a function of p_H; thus at p_H 3·5 ovalbumin retards reaction by about 5 %, whilst at higher p_H it accelerates reaction progressively to 43·4 % at p_H 7·5.

Table V. *Action of ovalbumin at varying* p_H.

	mg. maltose produced		
p_H	With protein	Without protein	% acceleration
3·5	50·3	52·8	−4·9
4·5	63·8	60·6	+5·3
6·0	61·7	55·1	+11·9
7·5	46·1	32·1	+43·4

2. *Influence of ovalbumin in 5 % alcohol.* Similar systems to those described in the preceding experiment were set up, with the difference that 3·125 cc. of alcohol were added in place of a similar volume of water, so that these systems contained 5 % of alcohol. The p_H, temperature and duration of reaction were as before. The results (Table VI) were of the same order as in the absence of alcohol; the absolute yield of maltose was, however, smaller in all cases (retardation due to alcohol), whilst the retarding or accelerating influence of ovalbumin was relatively greater (12·3 % retardation at p_H 3·5, and 77 % acceleration at p_H 7·5).

Table VI. *Action of ovalbumin at varying* p_H *in 5 % alcohol.*

	mg. maltose produced		
p_H	With protein	Without protein	% acceleration
3·5	45·1	51·5	−12·3
4·5	63·1	58·4	+ 8·1
6·0	56·4	48·5	+16·3
7·5	41·8	23·2	+77·0

3. *Influence of gelatin at varying* p_H. A number of flasks were prepared, containing 15 cc. each of 3 % starch, phosphate buffers, and 4 % gelatin solution or water, 14 cc. of water and 1 cc. of 0·1 % amylase. The systems were buffered to p_H 5, 6, 7 and 8, and were kept 1 hour at 30°, after which maltose was determined as usual. The results obtained (Table VII) are not of the same

Table VII. *Action of gelatin at varying* p_H.

	mg. maltose produced		
p_H	With gelatin	Without gelatin	% acceleration
5	59·0	54·9	7·3
	80·2	77·9	3·0
6	65·6	59·3	10·8
7	49·3	38·4	29·0
	47·0	41·9	12·7
8	40·0	28·8	60·1

order of accuracy as in other experiments, owing to the unreliable values given by Bertrand's method in the presence of gelatin, and to the circumstance that experiments at each p_H were conducted separately. It is, however, obvious that gelatin accelerates reaction increasingly from p_H 5 to 8.

4. *Influence of gelatin in 20 % alcohol.* The systems consisted of 15 cc. each of 3 % starch, phosphate or acetate buffers, and 4 % gelatin or water, 12·5 cc. of alcohol, 1·5 cc. of water and 1 cc. of 0·1 % amylase. The systems were at p_H 3·5, 5, 5·8, 6·5 and 7, and were maintained at 30° for 1 hour. As in the preceding experiment, each value is strictly valid only for the corresponding control. Gelatin acts similarly in alcoholic and aqueous systems, its effect being however more marked in the former. In particular, very considerable retardation (64·6 %) is observed at p_H 3·5 (Table VIII), whilst at higher p_H acceleration increases from about 10 % at p_H 5 to about 70 % at p_H 7.

Table VIII. *Action of gelatin at varying* p_H *in 20 % alcohol.*

	mg. maltose produced		
p_H	With gelatin	Without gelatin	% acceleration
3·5	13·6	38·7	−64·6
5·0	48·0	41·8	+13·3
5·8	47·7	37·6	+26·8
6·5	46·4	29·5	+57·3
7·0	30·0	17·2	+72·6

5. *Influence of peptone.* A number of systems consisting of 15 cc. each of 3 % starch, phosphate buffers to p_H 4·5, and 4 % Witte's peptone or water, 14 cc. of water and 1 cc. of 0·1 % amylase were kept 1 hour at 30°, and maltose was determined as usual. A mean value of 66·0 mg. was obtained in the presence of peptone, and of 60·3 mg. in its absence, representing an acceleration of 9·3 % due to peptone.

6. *Influence of glycine.* In the above experiments the effect on the action of amylase of addition of colloidal proteins or protein derivatives has been studied; the effects obtained have been ascribed by certain authors to protective colloidal action. In order to ascertain to what extent this view is justified, experiments were performed on the action of crystalloid protein derivatives.

(*a*) *Glycine.* A number of systems containing 15 cc. each of 3 % starch, buffer solutions, and 4 or 8 % glycine solution or water, 14 cc. of water and 1 cc. of 0·1 % amylase were kept 1 hour at 30° after which maltose was determined. The concentration of glycine in the systems was 0·133 *M* and 0·266 *M*. The results (Table IX) indicate that glycine has a similar action to that of the colloidal protein derivatives studied previously, and that the effect obtained increases with the concentration of glycine.

That the action of glycine is not exerted directly on the substrate was shown by experiments in which similar systems to the above, but without amylase, were taken; no production of maltose took place.

Table IX. *Action of glycine at varying* p_H.

Systems as in Table V, except that 4 or 8 % glycine is taken in place of 6 % ovalbumin.

	1 % glycine			2 % glycine		
	mg. maltose produced			mg. maltose produced		
p_H	With glycine	Without glycine	% acceleration	With glycine	Without glycine	% acceleration
3·5	51·1	54·2	− 6·4	—	—	—
4·5	69·4	61·0	+13·8	65·2	50·8	+28·4
6·0	65·5	54·3	+21·0	63·0	45·4	+38·8
7·5	48·3	35·1	+38·0	48·4	30·4	+59·2

(*b*) *Influence of temperature on action of glycine.* Systems of the same composition as in the above experiments, and at p_H 3·5, 4·5, 6 and 7·5, were studied at 18, 30, 38 and 50°. The time of reaction at 18° was 4 hours, and the yields of maltose were divided by 4 in order to obtain comparative results with systems kept 1 hour at 30, 38 and 50°. The results obtained (Table X) indicate that the absolute velocity of reaction increases up to 50°; at 60° the activity of amylase is smaller than at 50°. This is in accordance with Chrząszcz's finding [1923] that at the optimal p_H amylase exhibits maximal activity at 55°. At lower p_H values the velocity of reaction appears to be independent of temperature. At higher p_H the accelerating effect due to glycine increases with temperature.

7. *Influence of sodium aspartate.* Similar experiments to those with glycine were performed, substituting 15 cc. of 9·4 % sodium aspartate for the same volume of 4 % glycine, so that the molar concentration (0·133) was the same as for glycine. At p_H 4·5, 97·6 mg. of maltose were produced in the presence of sodium aspartate, and 71·0 mg. in its absence, an acceleration of 37·4 %,

Table X. *Influence of temperature on enzymic hydrolysis of starch in the presence of glycine.*

Systems as in Table V, except that 4 % glycine is taken in place of 6 % ovalbumin. Time of reaction: 1 hour.

		mg. maltose produced		
Temperature	p_H	With glycine	Without glycine	% acceleration
18°	3·5	19·0	20·3	− 6·4
	4·5	28·0	23·6	+14·4
	6·0	24·2	20·0	+21·0
	7·5	17·6	12·6	+39·7
30°	3·5	54·9	58·5	− 6·2
	4·5	74·6	65·7	+13·7
	6·0	69·4	56·7	+22·5
	7·5	53·6	38·6	+38·8
38°	3·5	60·9	65·8	− 7·4
	4·5	89·5	78·5	+14·0
	6·0	84·1	66·9	+25·7
	7·5	64·6	35·5	+82·0
50°	4·5	125·0	107·0	+16·8
	7·5	81·0	31·0	+161·0

whilst at p_H 6·0 the corresponding figures are 63·8 and 51·0 mg., and 21·8 % acceleration.

D. *The active group of amino-acids.*

1. *The carboxylic group.* Sherman and co-workers [1927], who investigated the influence of acids on the action of pancreatic amylase, found that at the optimal p_H the presence of acetic, phosphoric, boric and citric acids did not affect the velocity of hydrolysis. The effects of addition of oxalic and acetic acids will now be described. Systems containing 15 cc. each of 3 % starch, phosphate buffer p_H 7·5, and 9·36 % potassium oxalate solution or water, 14 cc. of water and 1 cc. of 0·1 % amylase were kept 1 hour at 30°, and reducing sugar was determined. Oxalate retards reaction under these conditions by about 8 %, the yield of maltose being 45·2 mg. in the absence of oxalic acid, and 41·6 mg. in its presence. The molar concentration of potassium oxalate was 0·133.

The concentration of sodium acetate taken in similar systems was 0·266 M, so that the concentration of carboxylic groups was the same in both cases. Acetate produces a relatively greater retardation of reaction, *viz.* 29 %, the yield of maltose falling from 45·2 to 32·1 mg.

2. *The amino-group.* The influence of amino-groups has been investigated by Caujolle and Molinier [1930], who found that at 50° aliphatic amines inactivated pancreatic, salivary and malt amylases. The hydrochlorides of these amines, on the other hand, activated pancreatic and salivary amylases, but were without effect on malt amylase. These authors do not, however, state whether the p_H of their systems was affected by the addition of amines, so that it is possible that the effects observed were due to change in p_H. Sherman and Naylor [1922] also report an inactivation of amylase by aliphatic and aromatic amines.

(*a*) *Guanidine carbonate.* This was taken at concentrations of 0·013 and 0·133 M, in systems consisting of 15 cc. of 3 % starch, 15 cc. of hydrochloric acid solution, 7·5 cc. of 0·54 or 5·4 % guanidine carbonate solution or water, 21·5 cc. of water and 1 cc. of 0·1 % amylase solution. The p_H of the systems was adjusted to 4·5 and 6 by addition of hydrochloric acid of the requisite strength, and maltose was determined after 1 hour at 30°. The yield of maltose in the control systems was 76 mg., whilst in the presence of guanidine only traces of sugar were found.

(*b*) *Diethylamine.* Diethylamine in concentrations of 0·133 M similarly totally inhibits reaction at p_H 7·5.

(*c*) *Asparagine.* Asparagine (1 %) in similar systems totally inhibits reaction at p_H 4·5.

(*d*) *Methylamine hydrochloride.* This salt (0·133 M) totally inhibits reaction at p_H 7·5, whilst at p_H 4·5 the yield of maltose is 58·0 mg. in its presence, as compared with 71·1 mg. in its absence, a retardation of 18·4 %. Sørensen's phosphate buffers were used in these experiments.

(*e*) *Urea.* 0·133 *M* urea has only a small retarding influence, the yield of maltose at p_H 4·5 being 71·0 mg. in its presence, and 75 mg. in its absence, a retardation of 5·4 %.

(*f*) *Choline hydrochloride.* This salt, at concentrations of 0·133 *M*, exerts a slight activating influence, the yield of maltose being 63·6 mg. in its presence, and 61·0 mg. in its absence, an activation of 4·3 %.

Discussion.

The above results indicate that proteins and their derivatives are capable of exerting a profound influence on the velocity of enzymic hydrolysis of starch, to an extent dependent on the p_H of the medium. At p_H lower than the optimal (p_H 4·5) the reaction is in all cases retarded, whilst at and above p_H 4·5 it is accelerated to an extent proportional to the alkalinity of the system.

These results appear to be connected with Przyłęcki and Majmin's [1931] work on the combination of proteins and their derivatives with starch. These authors found that the stability of such complexes varied inversely with the p_H of the medium, combination being irreversible in acid solutions. The starch in such complexes is possibly more resistant to the action of amylase than is free or loosely combined starch, and this might explain the inhibitory action of the substances examined in systems at p_H 3·5.

Activation of reaction at higher p_H may be due to activation of the enzyme or of the substrate, or to protection of the enzyme from some anticatalyst formed during the reaction; which of these factors is actually responsible for the effect observed it is not possible, on the basis of the experimental material available, to state. Since, however, the action of protein substances on the enzyme itself has not been examined, these explanations must be regarded as being purely speculative.

The magnitude of the effect observed appears to be proportional to the active concentration of amino-acid units present, as is shown by Table XI.

Table XI.

Substance	% concentration	p_H	% acceleration
Ovalbumin...	1·5	4·5	5·3
Gelatin	1·0	5·0	6·6
Peptone	1·0	4·5	9·0
Glycine 0·133 *M*	1·0	4·5	13·8
Glycine 0·266 *M*	2·0	4·5	28·4
Sodium aspartate 0·133 *M*	2·3	4·5	37·4

Percentage acceleration is twice as great at p_H 4·5 in presence of 2 % as in that of 1 % glycine. At other p_H the results are qualitatively of the same order, but the proportionality between concentration and activation is not so exact.

The action of amino-acid groups is not due to either the amino- or the carboxyl groups taken separately; the addition of carboxylic acids is either without effect or exerts a retarding action, whilst amines inhibit reaction to an

extent proportional to the basic character of the amino-group. These findings are in accordance with those of earlier workers in this field.

The action of proteins and of their derivatives appears to be due to their amino-acid units, and not, as has been supposed by certain authors, to their protective colloidal character. The results obtained possess a considerable biological significance, in that they indicate that the action of amylase within the living organism will be modified by the protein substances which are present in abundance, and this applies in particular to its action at varying p_H. Thus, in the intestinal digestion of carbohydrates it would follow that the velocity of hydrolysis should be greater when proteins are simultaneously undergoing digestion than when these are absent.

In the presence of ethyl alcohol, retardation of reaction is observed both in the presence and in the absence of protein substances, and this retardation is, at a given concentration of starch, roughly proportional to the concentration of alcohol, and is greater in alkaline than in acid media. On the whole, the action of alcohol is greater in more dilute than in more concentrated solutions of starch.

Were the inhibitory action of alcohol to be due to denaturation of the enzyme, it might be expected that this effect would be obtained in quite low concentrations of alcohol, as the concentration of enzyme is small. Experiment showed, however, that this is not the case, as the activity of the enzyme was little affected by 1 hour's contact with 20 % alcohol, and after 48 hours of contact alcoholic enzyme solutions were more active than were similar aqueous solutions.

The inhibitory action of alcohol may be due to some extent to diminution in the degree of dispersion of starch, solutions of which exhibit a marked turbidity on the addition of alcohol. This explanation would agree well with the observed proportionality between the concentration of alcohol and the percentage inhibition of reaction, as well as with the observation that the yield of maltose, at the same concentration of enzyme, is proportional to the concentration of the substrate only with small concentrations of starch. On the other hand, experiments with dextrin show that a practically identical inhibition of reaction is obtained as with starch, in spite of the fact that dextrin is aggregated by alcohol to a far less extent.

The possibility remains that alcohol acts on the enzyme-substrate complex, by eluting particles of starch from the surface of the enzyme, which is consequently isolated from contact with the substrate to a greater or less extent.

The effect of the addition of protein to systems containing alcohol is of the same order as in non-alcoholic systems, but is numerically greater, both at p_H at which protein retards and at which it accelerates reaction. The absolute values are, however, in all cases lower than in the absence of alcohol.

SUMMARY.

1. Ethyl alcohol retards the reaction of hydrolysis of starch by malt diastase to an extent proportional at p_H 5·6 to the concentration of alcohol. The optimal p_H in the presence of alcohol is the same as in its absence, *viz.* 4·5. Percentage retardation of reaction due to alcohol is greater the more the p_H of the system exceeds 4·5.

2. Similar results are obtained taking dextrin in place of starch.

3. The activity of 20 % alcoholic amylase solutions is greater than is that of similar solutions not containing alcohol, after keeping up to 48 hours.

4. Ovalbumin, gelatin, peptone, glycine and aspartic acid retard at p_H below the optimal (p_H 4·5), and accelerate reaction at p_H higher than 4·5, to an extent increasing with the alkalinity of the medium. In the case of glycine, the relative acceleration is greater the higher the temperature.

5. The action of protein substances is not due either to the carboxylic or to the amino-group separately, as both carboxylic acids and amines retard the reaction.

This research was undertaken at the suggestion and under the direction of Prof. St. J. Przyłęcki, to whom the author wishes to express his indebtedness for his help and supervision.

REFERENCES.

Caujolle and Molinier (1930). *Compt. Rend. Soc. Biol.* **104**, 294.
Chrząszcz (1923). *Biochem. Z.* **142**, 417.
Euler and Josephson (1926). *Z. physiol. Chem.* **152**, 31.
Przyłęcki and Majmin (1931). *Biochem. Z.* **240**, 98.
Sherman and Naylor (1922). *J. Amer. Chem. Soc.* **44**, 2956.
—— Caldwell and Adams (1926). *J. Amer. Chem. Soc.* **48**, 2954.
—— —— and Dale (1927). *J. Amer. Chem. Soc.* **49**, 2597.

CCII. SPERMINE IN HUMAN TISSUES.

By GEOFFREY ARTHUR HARRISON.

From the Department of Chemical Pathology, St Bartholomew's Hospital.

(*Received October 1st, 1931.*)

Although the structure of spermine has been elucidated by the work of Dudley, Rosenheim and their colleagues [1924, 1925, 1926], the physiological significance of this interesting base remains completely obscure. Originally observed in, and suspected as being specific to, human semen, subsequent investigation showed it to be a widely distributed constituent of mammalian tissues, both male and female.

That it is not essential to the process of fertilisation was made extremely probable by the failure of Dudley and Rosenheim [1925, 2] to detect it in the semen of the bull. An explanation of this striking difference from human semen is advanced in the present communication, which is concerned mainly with the distribution of the base in human tissues; it was considered desirable to make this survey in order to compare the results with those of Dudley and Rosenheim [1925, 2] for other mammalian tissues, and in the hope that the information obtained might eventually contribute to an understanding of the biological significance of the base.

Method of isolation.

The base was separated by the steam-distillation method [Dudley, Rosenheim and Rosenheim, 1924]. A number of minor modifications were introduced so that a description may be of value.

The organs, which were obtained from the *post mortem* room, were the seat of various pathological lesions. By working up one gland at a time it was hoped originally that variations due to different diseases might be revealed, at any rate in the case of the larger glands. In nearly all cases (for exceptions, see below), however, the yield of spermine was so low that it was necessary to use 5 kg. of minced organ to work quantitatively; for this reason the attempt to isolate spermine from a single organ had to be abandoned.

With the exceptions noted below, the following procedure was finally adopted. The organs were cleaned by dissection, minced, and stored under absolute alcohol, until 5 kg. or more of a particular organ had been collected. The fluid was then strained off, the alcohol was removed by distillation and the residue was added to the mince. The weight of the mince *plus* residue was determined, and the final yield was expressed as mg. of spermine phosphate per 100 g. of this material. These were obviously not the yields per 100 g. of fresh

organ (they were a little higher in most cases), but were satisfactory for comparative purposes. In the case of liver, however, all the observations were made on organs as observed at autopsy; in the case of lung and brain also it was sometimes possible to obtain enough minced organs to avoid storage under alcohol.

The 5 kg. of minced organ were added to 10 litres of boiling tap-water, and thoroughly mixed. After the addition of 30 cc. of glacial acetic acid and further stirring, the mixture was again boiled and kept simmering for half an hour. The coagulated protein was allowed to settle, and the supernatant fluid was syphoned off and filtered on a large Büchner funnel. The bulky mass of coagulated protein was transferred to a press, and as much fluid as possible was squeezed out and then passed through the Büchner funnel. The combined filtrates were then evaporated to about 500 cc. The contents of the pan were transferred, without filtration, to a glass vessel, together with a little wash-water. After introducing 25 cc. of glacial acetic acid, a saturated aqueous solution of normal lead acetate was added till there was no further immediate precipitate. The mixture was either filtered on a Büchner funnel or (more conveniently) passed through a Sharples centrifuge. The excess of lead was removed from the filtrate by cautious precipitation with 50 % *v/v* sulphuric acid. The lead sulphate was removed by filtration or centrifuging. The filtrate was made slightly alkaline to litmus by adding flake sodium hydroxide, and concentrated to about 400 cc. Ammonia was thus largely removed, but no spermine was lost.

The concentrate was transferred, with sufficient wash-water to make a total volume of 500 cc., to a 3 litre round-bottom flask of resistance glass, and 50 g. of flake sodium hydroxide were added. A grease-mark was made on the flask to indicate the level of the fluid which was maintained at constant volume. The mixture was steam-distilled in the usual way, a Kjeldahl trap being inserted in the exit tube to prevent loss of the solution by frothing or spitting. Frothing was often troublesome, and it was generally essential to regulate the distillation carefully for a few minutes, and sometimes to add a little vaselin.

Distillation was continued until there was no longer any smell of ammonia, and until the distillate was only slightly alkaline to litmus (usually 2 to 6 litres of distillate). In some cases the fluid which came over gave a positive Dragendorff's reaction, but it was shown that this was due to other bases and never to spermine, provided that the contents of the distilling flask were not allowed to become concentrated. (To make certain of this point, this "10 % distillate" was on many occasions worked up separately, but no spermine was recovered.) When a positive Dragendorff's reaction was obtained, distillation was continued until it became negative. This preliminary distillation from 10 % sodium hydroxide was a valuable means of getting rid of most of the ammonia (formed by the action of the alkali), and of some of the "lower bases."

The contents of the distilling flask were allowed to cool a little, and then a further 200 g. of flake sodium hydroxide were added. The steam-distillation was again started, and continued vigorously until samples of the distillate no

longer gave a positive Dragendorff's reaction. Care had to be taken that the contents of the flask did not become diluted by condensed steam. To ensure that the whole of the base had been distilled over, a further 50 g. of sodium hydroxide were added and the distillation was continued. This was repeated if necessary until Dragendorff's reaction was negative. As a rule some 5 to 15 litres of distillate were collected. This was made acid with concentrated hydrochloric acid and taken to complete dryness by distillation *in vacuo* or by evaporation on the water-bath. (Completion of the drying and the volatilisation of the excess of hydrochloric acid were facilitated by adding absolute alcohol in the last stages of evaporation.)

The very crude spermine hydrochloride thus obtained was dissolved in the minimum of distilled water and filtered with suction through a large filter-paper to remove a small amount of greasy pigmented matter, which was washed with distilled water. The combined filtrate *plus* washings was acid, and on making slightly alkaline to litmus by the cautious addition of 40 % sodium hydroxide, a further small pigmented precipitate was generally obtained and was filtered off after adding decolorising charcoal.

The final solution of spermine hydrochloride was treated with a concentrated solution of sodium picrate (picric acid 23 g., *N*/1 NaOH 100 cc., water 500 cc.) until no further precipitate could be obtained. After standing at room temperature for a few hours (generally overnight) the crude spermine picrate was filtered off, dried and weighed. (The mother-liquor, after concentration, as a rule yielded no significant quantities of spermine picrate.)

The dry crude picrate was suspended in sufficient acid alcohol (concentrated HCl 7 cc., water 43 cc., alcohol to 200 cc.) to dissolve it after heating on the boiling water-bath for about 10 minutes. For each 1 g. of picrate 15 to 25 cc. of the acid alcohol were generally required. The hot solution was poured into about 10 volumes of acetone, the glass vessel being washed out with a further few cc. of hot acid alcohol. The mixture was kept until the precipitate clumped and was then filtered with suction. The precipitate was washed with acetone until free from picric acid.

This precipitate of crude spermine hydrochloride rapidly deliquesced; the funnel was therefore promptly connected up with another filter-flask, a small volume of water was added and the solution sucked through. To this solution the calculated amount (weight of picrate $\times$ 0·236) of diammonium hydrogen phosphate, dissolved in a small volume of warm water, was added. An equal volume of absolute alcohol was added to the mixture which was kept at room temperature overnight and then filtered. The precipitate was dissolved in the minimum of boiling water and the solution treated with charcoal and filtered hot.

To the final filtrate which was colourless, a few drops of phenol red were added, and (if necessary) 10 % ammonia until the reaction was p_H 7·0 to 7·2 (warm brown tint). Crystallisation of spermine phosphate often started rapidly but was completed by adding $\frac{1}{3}$ vol. of absolute alcohol. After standing overnight at room temperature, the precipitate of spermine phosphate

was filtered off, dried and weighed. The mother-liquors after addition of alcohol to 50 % and to 25 % respectively, in the last two stages, were always worked up, but seldom yielded significant amounts of spermine phosphate.

Notes on method.

When the first filtrate, after removal of protein, contains appreciable quantities of fatty material in suspension, it is necessary to introduce one or more extractions with ether.

Treatment with lead may be omitted, as in the original description [Dudley *et al.* 1924], but if it is, considerable quantities of ammonia are formed owing to the action of sodium hydroxide on the protein, and frothing is likely to be much more troublesome. Filtration of the lead precipitates is in some cases very tedious or difficult; centrifuging is a great improvement.

If the steam-distillate is treated with phosphoric acid as described by Dudley *et al.* [1924], and is then evaporated to a small volume, it becomes very acid owing to loss of ammonia. If the reaction is not readjusted, spermine phosphate will generally not crystallise out at all. If concentration is effected before adjustment of reaction with phosphoric acid, spermine phosphate crystallises out, but in the writer's experience the yield is lower than in the method above described, possibly owing to the effect of the other phosphates on the solubility of spermine phosphate. The preliminary distillation after addition of sodium hydroxide to 10 % largely eliminates this difficulty.

The conversion of the crude hydrochloride (obtained by acidification and evaporation to dryness of the steam-distillate), into picrate, into hydrochloride and then into phosphate, may at first sight appear a tedious and unnecessarily complicated procedure, but gives a comparatively clean and pure preparation with more certainty, in the writer's opinion, than the original method of Dudley *et al.* [1924]. Incidentally it may be noted that the weight of picrate has generally been some four to ten times the weight corresponding to the yield of phosphate finally obtained (except in the case of the prostate). This presumably is due in part at any rate to the presence of picrates of bases other than spermine [*cf.* Dudley, Rosenheim and Starling, 1927].

A better yield was obtained by first precipitating crude spermine phosphate in the presence of 50 % alcohol and then recrystallising from 25 % alcohol, than by precipitating it initially with $\frac{1}{4}$ vol. of alcohol.

Finally, decoloration with charcoal of extracts, which are generally deep brown, allows phenol red to be used and therefore a close adjustment of reaction to be made.

Conversion of spermine into phosphate via *picrate and hydrochloride.*

In order to gain some idea of the magnitude of losses which might be expected in the process of conversion of crude spermine hydrochloride into picrate, into hydrochloride and finally into phosphate, the following experiment was performed on pure spermine phosphate[1].

0·4315 g. of spermine phosphate was suspended in 10 cc. of water and sufficient sodium hydroxide solution was added to cause solution (12 drops of 40 % NaOH). The solution was then treated with sodium picrate, and the subsequent stages were carried through exactly as described for a tissue extract.

The 0·4315 g. of phosphate gave 0·92 g. of picrate (theory, 0·953 g.), 0·285 g. of hydrochloride, 0·394 g. of "crude" phosphate, and 0·370 g. of once recrystallised phosphate. That is to say approximately 86 % of the original phosphate was finally recovered. Since this experiment further experience

[1] Kindly presented by Dr Dudley.

has been obtained in handling spermine salts, and it is now considered that this recovery should be regarded as a minimum with ordinary care.

Yields.

The yield of spermine, as phosphate, in mg. per 100 g. of tissue is shown in Table I. The result of each experiment is given, except in the case of liver on which eleven sets of analyses were made. The results of Dudley and Rosenheim [1925, 2] for certain animals are quoted for comparison.

Wrede [1926] separated spermine as the chloroaurate from several organs of man, cow and bull. It is probable that his chloroaurate contained small quantities of bases other than spermine; on the other hand the maximum weight of organ employed by him was 2 kg., which would tend to give low yields. His results (recalculated as spermine phosphate) for human testis (0·75), muscle (2·25), brain (3·75), spleen (5·75), liver (12·5), pancreas (13·5) and semen (95 mg. per 100 g.) are of the same order as the writer's (see Table I).

Table I.

	Spermine as phosphate, mg. per 100 g.	
Tissue	In man	In animals (Dudley and Rosenheim)
Semen	90, 100, 117, 260*	0 (bull)
Testis	1·2, 9·3	6 (bull)
Prostate	130	—
Ovary	—	14 (cow)
Pancreas	16·1	25 to 30 (ox)
Liver	2·3 to 11·4	16 (ox)
Kidney	5·0, 5·9	15 (ox)
Spleen	3·0, 8·0	11 (ox)
Lung	3·3, 3·3, 4·7	7 (ox)
Heart	1·5, 2·3	—
Muscle	—	12 (calf), 4 (sheep) 2 (ox), 2 (horse)
Brain	1·8, 2·0, 3·1	7 (ox)
Thymus	—	5 (calf)
Adrenal	—	5 (ox)
Thyroid	—	3 (ox)
Bone marrow	0	—
Blood	0, 0	0 (ox), 0 (sheep)†
Serum	0	0 (ox)
Sputum	0, 0	—
Faeces (dried)	Trace, trace	—
Milk	—	0 (cow)
Egg	—	0 (hen)

* Result of Rosenheim. † Personal finding, 7791 cc. sheep's blood.

Notes on Table. With the following exceptions 5 kg. or slightly more of tissue were used for each analysis: testis, 975 and 1551 g.; prostate, 1594 g.; pancreas, 3361 g.; whole blood, 400 and 600 cc.; serum, 250 cc.; sputum, 380 and 200 cc.; bone marrow, 15 cc.; dried faeces, 450 and 190 g. The testes and prostates took over a year to collect. Further details as regards the yields from semen are as follows: (*a*) 18·3 cc. collected under alcohol; steam-distillation method applied to precipitate; yield 90 mg. per 100 cc. (*b*) 4·9 cc. of fresh seminal plasma (spermatozoa removed by centrifugation); direct separation of Böttcher's crystals; yield 100 mg. per 100 cc. (*c*) 60 cc. semen, pooled, stored in ice-room for several weeks without preservative; steam-distillation from alkali applied direct to untreated semen; yield 117 mg. per 100 cc. In addition spermine phosphate (Böttcher's) crystals have several times been separated from whole semen, both fresh and stale, by careful adjustment of reaction, but not quantitatively.

Discussion of results.

The yields obtained for the majority of tissues need no special comment. Spermine is present in almost every tissue of man just as in animals [Dudley and Rosenheim, 1925, 2], but in most organs of man it is in slightly lower concentration.

The two points of chief interest arising out of the present investigation are first, the large yield of spermine from the prostate, and second the presence of the base in the faeces.

The prostate consists approximately of two parts of muscle and one part of glandular tissue. If it may be assumed that most of the spermine is derived from the glandular portion of the gland, then the yield of 130 mg. per 100 g. of whole gland represents a yield of 300 to 400 mg. per 100 g. of glandular tissue; in other words it makes it likely that the main part, if not the whole, of the spermine of human semen is derived from the prostatic secretion.

Bull's semen contains no spermine [Dudley and Rosenheim, 1925, 2]; the bull does not possess a true functional prostate, but only a rudimentary collection of compound acinar glands which are regarded as the equivalent of the human prostate. This may account for the absence of spermine from the semen of the bull. Wrede [1926] recovered 70 mg. of spermine chloroaurate from 600 g. of bull's "prostate," which is equivalent to a yield of 3·5 mg. of spermine phosphate per 100 g. of tissue. Whether the "Rinder prostata" used by Wrede was rudimentary prostate or seminal vesicles or both, is immaterial; his yield was so low (of the same order as found in other organs of the body) that it is compatible with the absence of spermine from bull's semen.

The writer's observation (see notes on Table I) that fresh human seminal plasma yields at least as much spermine as whole semen, makes it unlikely that the spermatozoa are a significant source of the base.

Fürbringer [1886] stated that human prostatic secretion contained an organic base, the phosphate of which constituted "Böttcher's crystals" or "prostatic crystals." It is only recently [Rosenheim, 1924] that "Böttcher's crystals" from human semen were shown conclusively to consist of spermine phosphate, but it is curious that no worker seems to have explored further Fürbringer's statement that in man "Schreiner's base" (as spermine was then called) was derived from the prostate. Fürbringer [quoted by Von Frisch, 1899] found that the prostatic fluid squeezed out of prostates obtained *post mortem* contained Böttcher's crystals in 56 cases (90 % of cases examined). Fürbringer failed to recover Böttcher's crystals directly from fresh prostatic secretion obtained by massage of the gland during life, but he found that the crystals could be made to appear by adding ammonium phosphate. He concluded therefore that the prostatic secretion supplied the base, and that the phosphoric acid was derived from other components of the semen. He explained his finding of Böttcher's crystals in *post mortem* prostatic fluid by assuming that autolysis of epithelium supplied the phosphoric acid.

Although only some 1 to 5 drops of prostatic fluid are usually obtained by massage during life, it is easy to confirm Fürbringer's experiment of producing Böttcher's crystals by adding ammonium phosphate, though the crystals are of the characteristic shape only if the final reaction of the mixture is close to p_H 7·0. The writer has several times confirmed Fürbringer's work both by adding ammonium phosphate and by adding phenol red (as indicator) and dilute phosphoric acid to p_H approximately 7·0 to expressed prostatic fluid. In both cases the addition of a little alcohol (about 0·1 vol.) facilitated crystallisation. In one instance the addition of acetic acid (and phenol red) produced a few crystals of the typical lenticular shape, so that Fürbringer's failure to obtain Böttcher's crystals from prostatic fluid expressed during life may in part at any rate have been due to failure to secure the correct reaction.

It has been claimed that the prostatic secretion has an activating action on the spermatozoa [see Fürbringer, 1886, and Von Frisch, 1899]. There is no doubt of the effect of adding prostatic secretion to spermatozoa which have previously not come into contact with such secretion; the spermatozoa remain active for a much longer period in the presence of the prostatic fluid. Conversely, human spermatozoa, which have been separated from semen by centrifuging and washing with 0·85 % NaCl, do not remain motile for nearly so long as the spermatozoa left in suspension in the (diluted) seminal plasma (an observation several times confirmed by the writer). But neither of these observations of necessity proves that the normal function of the prostatic secretion is to activate the spermatozoa, though they support the idea.

It was however considered to be worth while to try the action of different concentrations of spermine phosphate in physiological saline on the motility of human spermatozoa separated from semen and washed three times with 0·9 % NaCl. Equal volumes of the suspension of spermatozoa and of spermine phosphate in physiological saline were mixed; control observations were made without the spermine phosphate. It was found that spermine phosphate in a final concentration of 19, 13, 1·3 and 0·13 mg. per 100 cc. had no activating action on the spermatozoa as compared with the controls.

The discovery of spermine in human faeces may be of significance. Just as putrescine in faeces is derived from the putrefaction of the arginine (ornithine) component of proteins, so spermine might be derived from the putrefaction of an appropriate component of proteins, although no parent substance, capable of yielding it, has yet been observed in protein hydrolysates. In a preliminary observation the writer has obtained traces of spermine after incubating a mixture of a platinum loopful of normal faeces with 2000 cc. of 2 % Witte's peptone for 63 hours, and 7 days respectively, under both aerobic and anaerobic conditions. Control tests, in which inoculation with organisms was omitted, were negative. This subject is under investigation.

SUMMARY.

1. A description of the steam-distillation method of separating spermine from tissues is given, modifications having been introduced to obtain the base as phosphate more readily in a relatively pure state.

2. A study of the distribution of the base in human tissues shows that it is virtually the same as that found in animals by Dudley and Rosenheim.

3. The spermine content of the human prostate is remarkably high, and probably most of the spermine of human semen is derived from this organ.

4. The absence of spermine from the semen of the bull is probably to be explained by the absence of a true functional prostate in that animal.

5. Spermine occurs in human faeces.

The author is indebted to the Medical Research Council for a grant towards the expenses of this work.

REFERENCES.

Dudley and Rosenheim (1925, 1). *Biochem. J.* **19**, 1032.
—— —— (1925, 2). *Biochem. J.* **19**, 1034.
—— —— and Rosenheim (1924). *Biochem. J.* **18**, 1263.
—— —— and Starling (1926). *Biochem. J.* **20**, 1082.
—— —— —— (1927). *Biochem. J.* **21**, 97.
Fürbringer (1886). *Berl. klin. Woch.* **23**, 476.
Rosenheim (1924). *Biochem. J.* **18**, 1253.
Von Frisch (1899). Nothnagel's Pathologie u. Therapie, **19**. (II. Theil, III. Heft.) 11.
Wrede (1926). *Z. physiol. Chem.* **153**, 291.

CCIII. STUDIES ON THE METABOLISM OF ANIMALS ON A CARBOHYDRATE-FREE DIET.

III. EFFECT OF ALKALI ON THE SENSITIVITY TOWARDS INSULIN OF ALBINO RATS.

By ALEXANDER HYND and DOROTHY LILLIE ROTTER.

From the Physiology Department, University of St Andrews.

(*Received October 5th, 1931.*)

In a previous paper [Hynd and Rotter, 1931] it has been shown that the effect of a carbohydrate-free diet on the sensitivity to insulin varies with the species of animal employed for the experiment. Further, as the results obtained pointed to the conclusion that animals became resistant to insulin on diets which were capable of causing a lowering of the alkali reserve, the experiments now described were carried out with the object of obtaining further support for this view.

As Hawkins [1924] has shown that rabbits and guinea-pigs show a great variation in acid-base equilibrium, while rats, on the other hand, exhibit considerable constancy both in p_H and CO_2 content of the whole blood, the rat was the animal selected for our experiments. Moreover, as we have found that piebald rats, though fed on the usual stock diet, vary considerably in their sensitivity to insulin, all the experiments described in this paper were carried out on standard albino rats of 120–160 g. body weight. The basis of the diets employed was bread and milk for the carbohydrate-fed animals, and cheese for the carbohydrate-free animals. To these diets were added varying amounts of different salts as detailed below, and after certain periods the animals were tested for their response to insulin. The experiments were so arranged that the effect of the salts on the response to insulin could be observed simultaneously in carbohydrate- and non-carbohydrate-fed animals, as well as in control animals on a normal diet without the addition of any salts.

Experimental.

(1) *Effect of sodium bicarbonate on cheese-fed rats.* Seven normal rats, which had been receiving the ordinary stock diet of bread and milk, were transferred to a diet of cheese to which was added 0·12 g. $NaHCO_3$ per g. of cheese. After 1 week the animals were tested with insulin: none was resistant.

The diet was continued for another week with 4 of the rats, and again all responded to the injection.

(2) *Effect of potassium bicarbonate on cheese-fed rats.* An experiment similar to the above was carried out with 8 rats, the sodium bicarbonate being replaced by an equivalent amount (0·14 g.) of potassium bicarbonate. On testing the animals with insulin after 1 week on this diet, all were resistant, but after 2 weeks they were found to have lost their resistance.

As the animals in this and in the preceding experiment lost weight, the second dose of insulin, being proportional to the body weight at the time of injection, was somewhat smaller than the first dose, so that the loss of resistance to insulin is all the more striking.

(3) *Effect of sodium bicarbonate on rats previously rendered resistant to insulin by a cheese diet.* Seventeen normal rats were fed on cheese for a fortnight and proved to be resistant to the usual dose of insulin. Each animal was then fed with cheese containing 0·12 g. $NaHCO_3$ per g. After 2 days on this diet, one animal was tested with insulin and found to be partially resistant. After 3 days, 4 rats were tested and found non-resistant; after 4 days, 7 other animals were tested and found non-resistant, and after 5 days, 5 other rats were also found non-resistant to insulin.

After 3 days, therefore, all the rats responded to insulin, although the behaviour was not quite typical. In almost every case the animals became cold and limp, and convulsions if they occurred were not of a violent type. Moreover, administration of glucose did not bring about a rapid recovery, and 3 of the animals died.

Similar results were obtained in an experiment with mice.

(4) *Effect of potassium bicarbonate on rats previously rendered resistant to insulin by a cheese diet.* Eight rats rendered resistant to insulin by a cheese diet were then given cheese and an amount of potassium bicarbonate equivalent to the sodium bicarbonate used in the preceding experiment.

After 5 days on this diet all the animals were still resistant. Four of the rats were continued on this diet for 9 days and still failed to respond.

Similar results were obtained in an experiment with mice.

(5) *Comparison of effect of (a) $NaHCO_3$ and (b) $KHCO_3$ on the blood-sugar, muscle- and liver-glycogen, and liver-fat of cheese-fed rats.* Owing to the striking difference obtained in the response to insulin after 5 days with potassium bicarbonate, as described in Exp. (4), compared with that after 3–5 days with sodium bicarbonate in Exp. (3), it seemed advisable to know the blood-sugar level, the muscle- and liver-glycogen content, and the liver-fat content of the animals in the two series.

Accordingly, 4 rats were kept on a cheese diet for 15 days and assumed to be resistant to insulin, as it seemed inadvisable actually to test the animals, lest the glycogen distribution might be affected thereby. As in 32 experiments we have encountered only 3 rats which under this treatment did not become resistant to insulin, the assumption seemed justifiable. Two of the rats were then fed on cheese containing 0·12 g. $NaHCO_3$ per g. for 4 days, and the other two on cheese containing the equivalent amount of $KHCO_3$ per g. for the same

period. That is, the $NaHCO_3$ pair when analysed would have lost their resistance to insulin, while the $KHCO_3$ pair would still be insensitive.

The analyses (Table I) show no significant differences.

Table I.

Rat	Diet	Response to insulin	Blood-sugar %	Muscle-glycogen %	Liver-glycogen %	Liver-fatty acids %
1	Cheese + $NaHCO_3$	+	0·175	1·0	1·4	5·4
2	,, ,,	+	0·145	0·93	1·8	4·2
	Average...	+	**0·16**	**0·965**	**1·6**	**4·8**
3	Cheese + $KHCO_3$	–	0·122	0·79	0·87	3·7
4	,, ,,	–	0·16	0·94	2·60	4·9
	Average...	–	**0·141**	**0·865**	**1·73**	**4·3**

(6) *Effect of sodium bicarbonate on carbohydrate-fed rats.* (*a*) *Small dose of $NaHCO_3$* per os. Five rats from the stock cages were kept without food for 9 hours. Each animal was then given bread and milk containing 0·5 g. $NaHCO_3$, and 15 hours later was tested for its response to insulin.

Four of the rats showed typical symptoms, though the effect, compared with that on a control animal receiving no alkali, was somewhat delayed in its development. Further, after the administration of glucose the animals did not recover well, and one of them, although repeatedly injected with glucose, never recovered sufficiently to be able to take food and died in about 6 hours after the insulin injection. One rat did not react, and, as this animal was still resistant to insulin after the alkali had been continued for a week, it was considered to be abnormal.

(*b*) *Large dose of $NaHCO_3$* per os. Three rats were each given bread and milk containing 2 g. $NaHCO_3$, and as in the preceding experiment then tested with insulin. All responded in a normal fashion, though the effect was slightly delayed.

(*c*) *Subcutaneous injection of $NaHCO_3$.* Two rats on a bread and milk diet were injected subcutaneously with 2 cc. of 8 % solution of sodium bicarbonate, and ½ hour later given insulin. In both, typical symptoms ensued very rapidly, as in 1 hour after the insulin injection glucose had to be administered to relieve the convulsions, whereas a control animal which received a corresponding dose of insulin showed no outward sign of hypoglycaemia until 1½ hours later.

(*d*) *Effect of alkali when continued for 1–2 weeks.* To the ordinary daily ration of bread and milk 0·5 g. $NaHCO_3$ was added and fed to each of 4 rats. Four other rats each received an equivalent amount of $KHCO_3$, and 3 rats were given a larger amount of $NaHCO_3$, *viz.* 2 g. each daily.

The animals took a sufficient quantity of food and remained almost steady in weight. Meyer and Cohn [1911] state that the addition of potassium bicarbonate to the diet of a rat causes the animal to lose weight rapidly, while with sodium bicarbonate rats put on weight. No difference in this respect was detected in the animals fed on the two alkalis. The examination of the urine

collected was rendered of rather doubtful value, as, though the animals took the diet well, there was always a considerable amount of food scattered. The urine in every case was found to be strongly alkaline and to reduce Benedict's solution. In the case of the rats receiving the larger amount of $NaHCO_3$, small amounts of acetone bodies were detected.

After 1 week on these diets, the animals were injected with insulin and all responded in a more or less normal manner. The rats which had received $KHCO_3$ differed from those treated with the sodium salt in that, though they had more violent convulsions, they made more rapid and more complete recoveries. On the other hand, the animals which had received sodium bicarbonate made slow recoveries from the effects of the insulin injection, relapses were frequent, and 2 of the rats were found dead the following morning.

The experiment with the rats on the potassium bicarbonate was continued for a second week and the animals were re-tested with insulin. A normal response again resulted in every case, though the development of symptoms was slightly delayed.

Animals treated similarly to those just described were also employed for determinations of blood-sugar, muscle- and liver-glycogen and liver-fat. The average values obtained are given below in Table II, and for comparison values are quoted from our previous paper [Hynd and Rotter, 1930]. In addition, there is included the result of the following experiment. A rat, weighing 160 g. at the beginning of the experiment, was fed for 1 week on bread and milk to which were added 2 g. $NaHCO_3$ daily. A control animal of equal weight was fed for 1 week on bread and milk, so adjusted that the two rats were eating practically the same amount of food. Both were then starved for 24 hours before making the analyses.

Table II.

Daily diet contains	Duration of experimental feeding (weeks)	Period of fasting (hours)	Blood-sugar %	Muscle-glycogen %	Liver-glycogen %	Liver-fatty acids %
0·5 g. $NaHCO_3$	2	0	0·15	0·77	3·4	—
Control	2	0	0·16	0·77	3·4	3·4
2 g. $NaHCO_3$	1	0	0·14	0·78	2·6	2·4
Control	1	12	0·13	0·70	2·5	3·7
2 g. $NaHCO_3$	1	24	0·11	0·90	1·1	4·2
Limited ration	1	24	0·135	0·81	1·1	4·5

(7) *Effect of ammonium chloride on carbohydrate-fed rats.* Each of 4 rats was fed on bread and milk containing 1–2 g. NH_4Cl per day's ration. As the animals objected to this diet, the experiment was continued for only 4 days. At the end of this period the rats were tested for their response to insulin. Three of the animals were resistant but showed the early transitory sprawling similar to that previously described for rats rendered insensitive to insulin by a cheese diet. The fourth rat reacted to insulin. This animal had very severe convulsions, from which it made only a partial recovery though injected with the

usual amounts of glucose. It died suddenly 5½ hours after the administration of the insulin.

(8) *Effect of alkali on rats previously rendered resistant to insulin by feeding ammonium chloride.* Four rats were rendered resistant to insulin by feeding ammonium chloride as described in the preceding experiment. Two of the animals were then given bread and milk containing sodium bicarbonate (1·2 g. $NaHCO_3$ each daily). The other two received the equivalent of potassium bicarbonate. After 2 days the animals were no longer resistant to insulin. All responded in a normal manner, the symptoms being similar in both sodium- and potassium-fed animals.

Discussion.

Several years ago Murlin and Kramer [1913] suggested that mobilisation of glycogen is favoured by an acid medium and its storage by an alkaline medium. The results of the foregoing experiments are in full accord with this hypothesis and prove beyond question that the acid-base balance is an important factor in determining the effectiveness of the pancreatic hormone.

The resistance to the action of insulin, which rats develop when maintained on a carbohydrate-free diet, can be altered by the addition of alkalis to the diet. Animals previously rendered resistant to insulin by a diet of cheese display a more or less normal sensitivity after 3 days' treatment with sodium bicarbonate. On the other hand, an equivalent amount daily of potassium bicarbonate has little, if any, effect even though continued for 9 days. Nevertheless, analysis shows (Table I) that the blood-sugar, the muscle- and liver-glycogen and also the liver-fat of a rat giving a response to insulin after the sodium bicarbonate treatment are practically identical with those of a rat still resistant to insulin after the ingestion of an equivalent amount of alkali in the form of potassium bicarbonate. Again, while sodium bicarbonate prevents a cheese diet from bringing about resistance to insulin, potassium bicarbonate fed along with cheese produces resistance in a shorter period (1 week) than does cheese alone (12–14 days). Continued feeding of potassium bicarbonate, however, eventually destroys the resistance to insulin, so that it appears that this difference between the two salts is only temporary.

Bunge [1873] concluded that the ingestion of large amounts of potassium led to an increased output of sodium, so that though large amounts of salts were being fed, the output might exceed the intake and a lowering of alkali reserve result. However, even though the high intake of potassium were continued, a point would soon be reached at which the body would retain its sodium, so that the alkali reserve would rise. Our results are explicable on this theory. Feeding potassium bicarbonate would produce a temporary period of low alkali reserve, during which the animals would be resistant to insulin, and this would be followed by a gradual increase in the alkali reserve with a consequent return of a more or less normal response. Feeding sodium bicarbonate on the other hand, would lead to a steady increase in the alkali reserve, as no

drastic elimination of potassium results, and consequently no resistant period would be observed. Richards, Godden and Husband [1927] find, however, that large doses of potassium do not lead in growing pigs to an increased elimination of sodium, or to an increased retention of potassium, but cause an increased excretion of calcium. Excess of sodium, on the other hand, was followed by retention of calcium, so that the Na/K ratio of a diet may have a very considerable influence on the calcium assimilation. The fact that Brougher [1927] has shown that insulin increases the blood-calcium may, therefore, be of importance. However, though certain experiments of Geiger and Kropf [1930] led them to form the opinion that a diet with a high calcium content favoured carbohydrate metabolism—a view supported by clinical experience—decreased calcium assimilation may play only a minor part in bringing about the resistance to insulin observed in our experiments. A simpler, although not complete, explanation is that owing to the diuretic effect of the potassium salt there is a smaller retention of total base when potassium bicarbonate is given than when sodium bicarbonate is the alkali used.

Further, it is to be noted that only on the acid-forming cheese diet does this difference between the effects of sodium bicarbonate and potassium bicarbonate become apparent. When rats are fed on the normal diet of bread and milk the only difference detected in the effect of the two salts on the response to insulin is that, as already pointed out, the animals receiving sodium bicarbonate are less liable to have convulsions and recover more slowly and less completely from the insulin symptoms. Probably in this series of experiments sodium bicarbonate increases the alkali reserve more quickly than does the potassium salt, but this point was not investigated as we were concerned mainly with the effect on the response to insulin.

As indicated in the experimental part, with one exception all the rats on the carbohydrate-diet with the addition of either sodium bicarbonate or potassium bicarbonate reacted to the insulin injection. Our results, therefore, are contrary to the findings of Goldblatt [1927], who reported that alkali inhibits the action of insulin. In his experiments, this worker used rats and also a rabbit which was admittedly abnormal. As he did not state the amount of alkali fed to each animal, it remains uncertain whether we have repeated his conditions, and in any case Goldblatt did not report on the action of insulin on rats fed for more than one day with alkali. Neither after a small dose nor a large dose of sodium bicarbonate *per os*, for one or more days, did we find the action of insulin inhibited, and administration of alkali subcutaneously definitely hastened and accentuated the response to insulin. We also failed to confirm the remarkable and almost complete disappearance of glycogen from both liver and muscle noted by Goldblatt to occur as the result of feeding sodium bicarbonate to rats for 3–8 days. Our values for the liver- and muscle-glycogen in rats fed with sodium bicarbonate are somewhat lower than those of controls, but the diminution is not striking, and we attribute the slight decrease to the fact that animals do not take the normal amount of bread and

milk when sodium bicarbonate is added to it. In other words, we are merely studying, in such experiments, the effect of slight undernutrition. This is indicated clearly by the results summarised in Table II, from which we conclude that alkali feeding does not reduce the liver- or muscle-glycogen content of carbohydrate-fed rats.

Goldblatt's contention that alkalis interfere with the action of insulin appears to receive support from the work of Haldane, Wigglesworth and Woodrow [1924, 2]. It is to be noted, however, that, though these workers found the ingestion of alkali to cause hyperglycaemia and glycosuria after less than half the normally tolerated dose of glucose, they did not actually demonstrate resistance to insulin. Moreover, the prolonged hyperglycaemia observed is not necessarily an index of defective insulin action [Cajori, Crouter and Pemberton, 1925], and further, Haldane and his co-workers draw attention to the fact that the symptoms of faulty sugar metabolism do not come on until long after the concentration of sodium bicarbonate in the blood is at its maximum. Goldblatt, further to support his view, also quotes the experiments of Hetényi [1926]. The results obtained by this investigator, however, not only in animal experiments but also with diabetic patients [Hetényi, 1927] point decidedly in the opposite direction, namely that alkali favours the action of insulin. Again Goldblatt's reference to the work of Koehler [1926] is misleading. While it is true that Koehler found huge doses of insulin to have no marked effect in several diabetic patients with marked acidosis (blood-p_H 6·8–7·2), Goldblatt omitted to add that "after alkali in the form of 3 % sodium bicarbonate solution was administered intravenously in just sufficient quantities to return the blood reaction to normal, the blood-sugar decreased very rapidly." Hédon [1925] also, as the result of experiments with a depancreatised dog, came to the conclusion that insulin and sodium bicarbonate are together required to restore the alkali reserve and health in cases of diabetes. Moderate dosage of sodium bicarbonate is also approved of by Campbell and Macleod [1925].

On the other hand, as it has been proved that insulin increases the alkali reserve, it is held by many and emphasised particularly by Joslin, that it is not only unnecessary but harmful to give alkali in diabetes. However, this assumes that the insulin is exerting its effect. If the conditions are such that the action of insulin is weakened in a marked degree, insulin treatment alone evidently will not correct the alkali reserve. In other words, it is essential that the alkali reserve to begin with be not too far reduced, otherwise the secreted or injected insulin will be rendered ineffective.

Further support for the view that the action of insulin is favoured by alkali and inhibited by acid is furnished by our experiments with ammonium chloride. The addition of this salt to the bread and milk diet very quickly produces in rats a severe acidosis, and even though abundant carbohydrate is supplied in the diet, there is established simultaneously a marked resistance to insulin, which, moreover, is easily corrected by replacing the ammonium chloride with either sodium or potassium bicarbonate, although here again the sodium salt

is more effective. At the same time, it must not be forgotten that an ammonium chloride acidosis is accompanied by an excessive excretion of water and also of sodium and potassium ions [Haldane *et al.*, 1924, 1]. Moreover, though the absolute loss of potassium may be less than that of sodium, the relative loss of potassium is much greater than that of sodium. Consequently, the Na/K ratio may be altered greatly. This may have an important effect on the action of insulin, especially as it is here shown that there is, for a time at least, a marked difference in the effects produced by sodium and potassium bicarbonates, both as regards their ability to dispel resistance to insulin and also in the type of insulin response which may be elicited.

In dealing with the resistance to insulin reference should perhaps be made to the well-known fact that fat depancreatised animals are much more difficult to control with insulin than are lean animals. This appears to us not to be due to a hyperglycaemia being maintained by a continuous conversion of fat into carbohydrate [Macleod, 1930], but rather to the tendency which fat animals show to develop acidosis and thereby render the insulin less effective. A similar explanation, that is, a greater tendency to acidity, seems applicable to the so-called resistant types of human diabetes. Lawrence [1927] holds that such cases of diabetes are either infected ones, or complicated by over-activity of the thyroid or suprarenals. While admitting that these glands are recognised antagonists to the pancreas, the conditions indicated are just those which would be accompanied by excessive acidity.

In conclusion it may be pointed out that the results of the present investigation suggest an alternative explanation to that usually given as to why glucose produced endogenously appears less stimulating to insulin production than ingested carbohydrate [Lawrence, 1926]. There is a large body of evidence to support the view that a raised blood-sugar is the normal stimulus to the production of insulin by the pancreas. Why then does the hyperglycaemia occurring in the later stages of starvation, when the metabolism of protein increases [Morgulis and Edwards, 1924], or that which results when a normal animal is maintained on an excess fat diet not stimulate the output of insulin and thereby correct the hyperglycaemic condition? The answer seems evident, for the formation of glucose from sources other than carbohydrate involves the simultaneous production of acid substances. Thus, though the glucose produced furnishes the necessary stimulus to the pancreas and an adequate secretion of insulin occurs, the acid character of the medium will interfere with the effectiveness of the insulin, and, at the same time, as shown by Abderhalden and Wertheimer [1924], will facilitate the action of adrenaline. The net result therefore would be an increased blood-sugar by a further depletion of the glycogen stores. The present tendency to increase the amount of carbohydrate and diminish the amount of fat in the diet of diabetics [Rabinowitch, 1930: Poulton, 1931] is thus shown to be founded on a sound basis. Further, as patients so treated keep well and require much less insulin, the clinical results support the views advanced in this communication.

SUMMARY.

Rats previously rendered resistant to insulin by a cheese-diet soon become responsive after the addition of sodium bicarbonate to the diet, but remain resistant after the ingestion of the equivalent amount of potassium bicarbonate.

Sodium bicarbonate is also more efficient than potassium bicarbonate in preventing the development of resistance to insulin in rats on a carbohydrate-free diet.

This striking difference between the effects of the bicarbonates of sodium and potassium is shown to bear no relationship to the carbohydrate stores of the animal. The acceptance of Bunge's theory of sodium impoverishment by potassium feeding furnishes a possible explanation.

Administration of sodium bicarbonate does not reduce the liver- or-muscle-glycogen of carbohydrate-fed rats, and it favours rather than inhibits the action of insulin.

An ammonium chloride acidosis is accompanied by marked resistance to insulin, which readily disappears after the addition of either sodium bicarbonate or potassium bicarbonate to the diet.

The results, taken as a whole, suggest that, even with abundant carbohydrate to stimulate the production of insulin and presumably with a normal secretion of insulin, the activity of the pancreatic hormone will be interfered with if the medium be too acid.

The clinical importance of the results obtained is discussed.

The authors are indebted to Prof. Herring for his continued interest in this work, and to the Carnegie Trust for research grants.

REFERENCES.

Abderhalden and Wertheimer (1924). *Pflüger's Arch.* **206**, 451.
Brougher (1927). *Amer. J. Physiol.* **80**, 411.
Bunge (1873). *Z. Biol.* **9**, 104.
Cajori, Crouter and Pemberton (1925). *J. Biol. Chem.* **66**, 89.
Campbell and Macleod (1925). Insulin, p. 110. (Williams and Wilkins, Baltimore.)
Geiger and Kropf (1930). *Arch. exp. Path. Pharm.* **147**, 272.
Goldblatt (1927). *Biochem. J.* **21**, 991.
Haldane, Wigglesworth and Woodrow (1924, 1). *Proc. Roy. Soc. Lond.* B **96**, 1.
—— —— —— (1924, 2). *Proc. Roy. Soc. Lond.* B **96**, 15.
Hawkins (1924). *J. Biol. Chem.* **61**, 147.
Hédon (1925). *Compt. Rend. Acad. Sci.* **180**, 545.
Hetényi (1926). *Klin. Woch.* **5**. 800.
—— (1927). *Z. ges. exp. Med.* **57**, 409.
Hynd and Rotter (1930). *Biochem. J.* **24**, 1390.
—— —— (1931). *Biochem. J.* **25**, 457.
Koehler (1926). *J. Biol. Chem.* **67**, xlv.
Lawrence (1926). *Quart. J. Med.* **20**, 69.
—— (1927). *Brit. J. Exp. Path.* **8**, 1.
Macleod (1930). *Brit. Med. J.* i, 918.
Meyer and Cohn (1911). *Z. Kinderheilk.* **2**, 360.
Morgulis and Edwards (1924). *Amer. J. Physiol.* **68**, 477.
Murlin and Kramer (1913). *J. Biol. Chem.* **15**, 365.
Poulton (1931). *Proc. Roy. Soc. Med.* **24**, 1291.
Rabinowitch (1930). *Can. Med. Assoc. J.* **23**, 489.
Richards, Godden and Husband (1927). *Biochem. J.* **21**, 971.

CCIV. NOTES ON OIL DEVELOPMENT IN THE SEED OF A GROWING PLANT.

By JOHN VARGAS EYRE.

(*Received October 9th, 1931.*)

Some years ago, in connection with problems arising in the Linen Industry, an examination was made of certain biological changes which take place in the flax plant during the processes of growth and of ripening. These had to do with the study of so-called "quality" of fibre for spinning and with "quality" of seed for special purposes. In the course of these investigations, results were obtained of considerably wider interest than those in view at the time, and although reference has been made to these investigations [Armstrong, 1924] the results have not hitherto been published. Through the courtesy of the Linen Industry Research Association it is now possible to give an account of this work.

The only previous work bearing on this question was that described by Ivanoff [1912] who examined the biological aspect of oil accumulation. This work was repeated under far more exactly controlled conditions by Eyre and Fisher [1915] who confirmed the general conclusions of Ivanoff and showed clearly that oil accumulates rapidly during the early stages of seed development, after which further increase is comparatively slight. During that year, in collaboration with Morrell [1915], who drew attention to the commercial significance of this work, attempts were made to trace changes in the chemical characteristics of this oil as the seed developed and matured, but the difficulty of obtaining, at any particular stage of development or ripeness, sufficiently large and uniform quantities of seed was not overcome and the results, although of considerable interest, could only be regarded as tentative. The flax plant continues to flower day by day over a long period; the individual flowers last but a few hours; they are self-fertilised, after which, generally about noon, the petals fall and from then onwards the seed develops and ripens.

On resuming this work with Barker during 1921 and 1922 special precautions were taken to raise a perfectly uniform crop of flax and each season more than 8000 flowers were individually marked at stated intervals to provide the requisite quantity of seed for a fuller examination. The variety of flax grown in 1921 was that known commercially as Blue Flowering Dutch. Warm fine weather prevailed during the whole period of the trials. In 1922 two varieties were grown, namely the tall fibre-bearing blue-flowering *Linum usitatissimum* from Riga and *Linum cribrosum*, a short seed-bearing variety

from Madrid. Cool, generally wet, conditions prevailed and the season was fully two weeks later than in 1921. The plots were maintained in such good order as to ensure uniform development and ripening over the whole area.

In order to avoid oxidation all samples of seed collected were at once removed from the capsule and dried at 100° in a current of dry carbon dioxide, a procedure which avoided the possibility of after-ripening of the seed and the undesirable accumulation of water in the light petroleum used for the oil extraction. The following method was employed for determining the amount of oil present in the dried samples of seed taken from time to time. The weighed sample was ground with purified sand, transferred to a Soxhlet thimble and extracted with light petroleum (B.P. 50° to 70°) for 8 hours, taking care that the whole process was conducted in a current of dry carbon dioxide. The iodine values of these extracted oils were determined under the usual standard conditions by means of Wijs's solution, comparisons being made with a linseed oil of known iodine value.

In 1921 the first sample was picked 6 days after marking (flowering) and was found to contain only a trace of oil[1]. Subsequently 26 further samples were taken at short intervals over a period of 53 days after flowering. The seeds themselves when examined within the capsule at a very early stage of development appeared white and almost translucent. About the fifth day, as chlorophyll made its appearance in the young seeds, they assumed a greenish hue which increased to deep green by about the eighteenth or twentieth day. After this stage had been reached, when the seeds were fully plump and well grown, the green colour gradually disappeared and a browning set in, until, by about the fortieth day after flowering the seeds were a full brown colour, becoming darker as the seeds ripened. The analytical results of examining the seeds and the oil extracted therefrom are shown graphically as curves *A*, *B* and *C* in

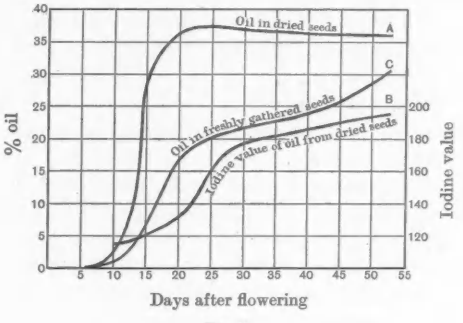


Fig. 1.

Fig. 1. On reference to these it will be seen that the synthesis of oleaginous extractable matter begins almost immediately after flowering and proceeds

[1] *I.e.* matter extractable by light petroleum.

comparatively slowly during the first 11 days. At the end of that time a rapid accumulation of oil sets in, amounting to about 3 % per day for some 10 days, when, after about 21 days from the time of flowering, the maximum oil content is reached and ceases with the disappearance of chlorophyll. As will be seen, observations were extended over a further period of 32 days, but during that period the variations in oil content were practically negligible when allowance is made for experimental errors due to sampling. Similar results were obtained with the two varieties grown in 1922, as will be seen on reference to the full line graphs shown in Fig. 2.

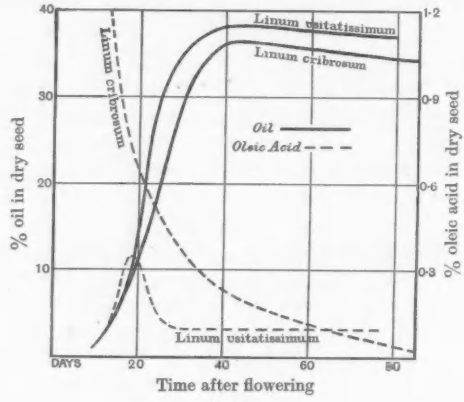


Fig. 2.

Changes in the character of linseed oil during seed development and ripening.

(i) *The colour of linseed oil.* During the course of this work wide variations were observed in the character of the linseed oil extracted at the various stages of development and ripeness of the seed. At first the oil was more than usually viscous and was of a greenish hue, but this gave place to a greater mobility and the characteristic full golden yellow colour as the age of the seed advanced. Further the oil became increasingly lighter in colour until, after 53 days from flowering, the seed yielded a very pale yellow oil and, when the harvested plants themselves, or merely the capsules containing the seeds, were stored under cover, indoors, the oil extracted from the seed was practically colourless.

(ii) *The unsaturated character of linseed oil.* In the case of a crop raised from *L. usitatissimum*, Dutch seed, the young seeds taken from the capsules when only 10 days old were found to yield an oleaginous residue having an iodine value of 114. Difficulties which attend the isolation of a sufficient quantity of this extract from seeds less than 10 days old prevent great reliance being placed upon analytical results obtained with such material, but it appears that the iodine value may well be as low as 100.

The iodine value of a good linseed oil varies between 180 and 200. On reference to Graph *B* in Fig. 1 it will be seen that the lower limit was reached after about 35 days, whilst the oil from seed 53 days old gave an iodine value of 191·9.

From a comparison of the graphs *A* and *B* in the same figure there appears to be no evidence of a change in the oil content parallel with the change in the iodine value of the oil. The interesting fact is revealed that the greatest rate of increase in the iodine value takes place after the oil content has attained its maximum value. The iodine value does not reach a maximum, but rises throughout the whole period during which observations were made, namely for 53 days after flowering, although the rate of increase diminishes somewhat towards the end of the period. The unsaturated character of the oil develops to a marked degree after the oil has been synthesised and the oil does not, as was generally supposed, become more and more saturated in character as the seed develops. Indeed, evidence points to the probability that changes in the degree of saturation are independent of those processes conditioning oil formation. Rapid oil formation occurs during those early days, 0 to about 20, when the colour both of the seed and of the extracted oil denotes the presence of chlorophyll; after that period, when the seed assumes a yellowish brown and full brown colour and the oil a full deep yellow colour, the iodine value of the oil rises rapidly, although no further increase in the oil content takes place. This view is supported by the following observed changes in the oil derived from seed when allowed to ripen under different conditions. It appears probable that these changes in the colour and iodine value of the oil are connected in some way either with residual life processes which persist in the plant, or are a separate set of changes controlled possibly by the carotene which persists throughout this period. Whether carotene is able to act so vigorously as an oxygen carrier as to remove hydrogen from the glyceride is not known, but from evidence available this does not seem improbable. The carotene itself may become oxidised during the final stages and thus account for the gradual loss of colour of the oil. In Table I will be found data relating to:

Material (*a*). Seed derived from detached capsules freshly gathered.

Material (*b*). Material as in (*a*) but set aside in the laboratory for varying periods.

Material (*c*). Seed derived from capsules not detached, but allowed to remain on the living plant while material (*b*) was after-ripening in the laboratory.

(iii) *The acidity of linseed oil.* The oils and fats which a plant produces from simple carbohydrates and stores as a reserve food material in its seed generally make their appearance as the seed approaches maturity, and, on germination, such reserve food is again converted into carbohydrates to support the young plant. In flax, however, it appears that oil formation sets in at an early stage, that the nature of the oil progressively changes, becoming more and more unsaturated in character, and contrary to expectations oil from fully mature seed is of greater value as a drying oil than is oil from less mature seed.

Table I.

Material (*a*) Seed from detached capsules freshly gathered			Material (*b*) Seed from detached capsules as (*a*) but after remaining at room temperatures for various periods						Material (*c*) Seed from capsules not detached from plant but allowed to remain on living plant while material (*b*) was at room temperatures				
Days after flowering	Oil %	Iodine value of oil	No. of days after (*a*)	Total days after flowering	Oil %	Increase of oil content %	Iodine value	Increase of iodine value	No. of days after flowering	Oil %	Increase of oil content %	Iodine value	Increase of iodine value
15	27·85	124·7	5	20	33·25	5·40	128·5	3·8	20	35·98	8·13	129·8	5
15	27·85	124·7	15	30	34·59	6·74	145·6	20·9	30	36·9	9·1	172	47
20	35·98	129·8	5	25	36·12	0·14	149·5	19·7	25	37·0	1·0	162	32
20	35·98	129·8	15	35	36·08	0·10	154·6	24·8	35	36·7	0·7	179	49
29	36·56	171·9	4	33	36·86	0·30	173·7	1·8	33	36·8	0·3	176	4

Glycerol, a substance closely related to the simple carbohydrates (sugars) from which presumably these compounds are formed, has been thought by some investigators to be the first product in the natural formation of linseed oil, the fatty acids being produced at a much later stage. Ivanoff, on the other hand, has suggested that in the synthesis of fats the higher saturated fatty acids are the first to be formed from the carbohydrates and that they are in turn converted into unsaturated acids which then combine with the glycerol present to form the fat.

It is of importance to have definite information on these points because for most technical purposes, as in the case of paint and varnish making, linseed oils of low acidity are highly esteemed. Should acid formation take place in the main during a definite period of seed development or ripening the character of the resulting oil may be in this respect influenced materially by harvesting the seed at a particular time. With the object of gaining information on these points in addition to following the course of oil formation, the acidity was measured of the oleaginous product obtained by extracting with light petroleum seeds of different degrees of ripeness. This was done by titrating with standard NaOH a solution of the oleaginous extract in neutral 90 % alcohol free from carbon dioxide, using phenolphthalein as indicator. The substances which are extracted from very young seeds by light petroleum are obtained as a solid or semi-solid mass when the solvent is evaporated. This extracted material is greenish-yellow in colour, readily becomes fluid when slightly warmed and is decidedly acidic.

On reference to Table II, columns 11, 12 and 13, it will be observed that as the seed of *L. usitatissimum* (Riga) ages the acidity of the oil diminishes rapidly, finally becoming almost constant at the low value of approximately 0·6. Commercial Baltic oil has an acidity of approximately 3 to 4 and Calcutta oil approximately 1 to 2. In the case of *L. cribrosum* (Madrid) similar results are recorded in Table III, columns 11, 12 and 13, from which it will be seen that in this variety of flax the oleaginous extract from the very young seeds is far more acidic than is the case with the *L. usitatissimum*, although it finally reaches a somewhat lower acid value. In this variety also the light petroleum extract from seeds 10 to 14 days old was obtained as a solid mass at ordinary

Table II. Linum usitatissimum (*Riga*).

	Freshly gathered seeds									Oil from dry seeds		
				Calculated for 1000 seeds			Dry seeds					
Age days	Wt. of 1000 seeds g.	Volatile at 100° %	Oil %	Vol. at 100° g.	Oil g.	Carbo-hydrates and protein g.	Wt. of 1000 seeds g.	Oil %	Colour	Acid value of oil*	Acid value (calculated) as oleic acid†	Remarks
1	2	3	4	5	6	7	8	9	10	11	12	13
12	6·3637	82·57	0·383	5·2545	0·0244	1·0848	1·1092	2·20	Greenish	—	—	Oil semi-solid at ordinary temperatures; oil fluid but of high viscosity; becoming less viscous as green colour disappears; oil much less viscous; marked decrease in viscosity and oil more readily oxidised
14	7·3264	80·88	0·553	5·9256	0·0405	1·3603	1·4008	2·89	yellow	8·6	43·428	
16	7·2771	78·17	1·207	5·6885	0·0878	1·5008	1·5886	5·53		8·4	41·454	
18	7·6685	76·89	2·078	5·8963	0·1594	1·6128	1·7722	8·99		7·8	38·493	
19	7·5838	74·63	2·935	5·6598	0·2226	1·7014	1·9240	11·57	Changing	4·8	24·083	
20	7·9179	74·92	4·015	5·9321	0·3179	1·6679	1·9858	16·01	gradually	4·2	20·727	
23	7·7178	68·89	7·267	5·3168	0·5609	1·8401	2·4010	23·36	from green	2·2	10·857	
25	7·6743	63·22	10·751	4·8517	0·8251	1·9975	2·8226	29·23	to deep	1·0	4·935	
27	7·5928	59·63	13·140	4·5276	0·9977	2·0675	3·0652	32·55	yellow	0·7	3·553	
30	7·7642	57·69	14·707	4·4688	1·1392	2·1382	3·2774	34·76		0·6	3·158	
32	8·1220	55·42	16·062	4·5012	1·3046	2·3162	3·6208	36·03	Green	0·6	2·961	
38	8·0422	47·92	20·254	3·8538	1·6289	2·5595	4·1884	38·89	disappeared	0·56	2·763	
46	7·6621	38·32	23·550	2·9361	1·7891	2·9369	4·7260	38·18	Pale yellow	0·60	2·961	
66	6·7209	28·70	26·673	1·9289	1·7927	2·9993	4·7920	37·41	Very pale	0·52	2·566	
76	6·3032	24·03	28·443	1·5147	1·7928	2·9957	4·7885	37·44	yellow	0·48	2·369	

Table III. Linum cribrosum (*Madrid*).

10	4·8426	84·43	0·11	4·0886	0·0053	0·7487	0·7540	0·68	Deep	—	—	Oil solid or semi-solid at ordinary temps.; oil fluid but of high viscosity becoming less viscous as green colour disappears
14	7·0844	81·76	0·56	5·7922	0·0397	1·2545	1·2922	3·08	greenish	83·0	420·462	
15	7·5610	79·77	0·83	6·0314	0·0628	1·4668	1·5296	4·09	yellow	—	—	
17	7·6892	77·35	1·46	5·9476	0·1123	1·6293	1·7416	6·45		23·3	118·045	
20	7·8856	75·88	2·44	5·9836	0·1924	1·7096	1·9020	10·11	More	14·2	72·248	
23	8·0137	72·19	4·62	5·7851	0·3702	1·8584	2·2286	16·62	intensely	7·0	35·532	
28	8·3667	67·71	8·97	5·6651	0·7505	1·9511	2·7016	27·79	green	3·2	16·582	
32	7·9594	60·60	13·07	4·8234	1·0403	2·0957	3·1360	33·18	Faintly	—	—	
36	7·6480	53·07	16·83	4·0588	1·2872	2·3020	3·5892	35·87	green	1·4	6·909	
44	8·6093	45·23	19·76	3·8940	1·7012	3·0141	4·7153	36·07	Green	1·1	5·525	Oil much less viscous; decrease in viscosity. Oil more readily oxidised
52	7·9823	41·16	21·14	3·2855	1·6875	3·0093	4·6968	35·93	absent	0·8	4·343	
66	7·3620	35·27	22·74	2·5966	1·6741	3·0913	4·7654	35·13	Very pale	0·7	3·553	
73	6·9107	31·90	24·01	2·2045	1·6593	3·0469	4·7062	35·26	yellow	0·18	0·928	
84	5·6634	16·81	29·32	0·9520	1·6605	3·0509	4·7114	35·24	oil	0·12	0·592	

* KOH per 1000 g. of oil. † Calculated as oleic acid per 1000 g. of oil.

temperatures. It is noteworthy also that this material was found to consist entirely of free acids, no indication being found of the presence of glycerides or other hydrolysable substances. It seems clear therefore that fatty acids are stored in the seed in the first instance, from which, presumably, the glycerides constituting the linseed oil are formed at a later stage. In Fig. 2 will be seen recorded graphically as full line curves the rate of development of the oleaginous extract or oil for the two varieties referred to, and as broken curves the corresponding acid development expressed in terms of oleic acid, as a convenient unit of acidity. The trend of these curves shows clearly how widely different the rates of acid formation in the seed of different varieties of flax may be, although the rate at which the oleaginous extract is stored in the seed is approximately the same in both varieties.

Through lack of material the lipase activity of the seeds at different stages of growth was not followed; it should certainly be done as this will probably throw interesting light on the mechanism of natural oil synthesis. If one may judge, however, from observations made regarding the activity of linase and the incidence of linamarin in the foliage and seeds of the same genus [Armstrong and Eyre, 1912] one may expect lipase activity to be coincident with glyceride formation.

Summary.

A study has been made of changes which take place in the rate of formation and in the character of the oil in the seed of flax—*Linum usitatissimum* and *L. cribrosum*—throughout its development and ripening period.

Observations show that in oil formation the acidic constituents are formed first. Whether the glycerol is formed at the same time, only combining with the fatty acid at a later stage, or whether the formation of glycerol itself is delayed is not clear. The observations reveal a remarkably rapid oil formation over a period of some 15 days, during which a maximum of about 36 % of oil, calculated on the dry weight of the seeds, is reached.

Changes in the nature of the oil continue after oil formation has ceased; its unsaturated character, as measured by its iodine absorption, continues to increase. It is pointed out that this is apparently an entirely separate change, independent of oil formation.

The interesting point is brought out that whereas during the early stages a reducing system must prevail in order to account for the formation of acids from carbohydrates, this must change to an oxidising system leading to the formation of unsaturated acids.

REFERENCES.

Armstrong and Eyre (1912). *Proc. Roy. Soc. Lond.* B **85**, 370.
Armstrong (1924). *J. Soc. Chem. Ind.* **43**, 207 T.
Eyre and Fisher (1915). *J. Agric. Sci.* **7**, 120.
Ivanoff (1912). *Beihefte Bot. Centr.* **28** (i), 159.
Morrell (1915). *J. Soc. Chem. Ind.* **34**, 109.

CCV. THE HYDROLYSIS OF CONCENTRATED SUGAR SOLUTIONS BY INVERTASE.

By HAROLD ALLDEN AUDEN and EDWARD ROMER DAWSON.

From the Research Department of the Distillers Company, Ltd., Epsom.

(*Received October 2nd, 1931.*)

Most of the extensive literature dealing with the kinetics of invertase action refers to the hydrolysis of dilute sucrose solutions. The commercial preparation of invert sugar from sucrose by hydrolysis in the presence of invertase necessitates the use of concentrated sugar syrups, and the object of the experiments reported was to study the relation between the rate of inversion and the sugar concentration.

Nelson and Schubert [1928] found that the velocity of hydrolysis by invertase decreased when the substrate concentration was greater than 10 %, the relation between sucrose concentration and velocity being approximately linear between 10 % and 70 % sucrose. This decrease in velocity was shown to be due to the falling off in water content.

Colin and Chaudun [1922] determined the relative viscosity of solutions containing from 10 to 60 g./100 cc. of sucrose and showed that the amount of sugar hydrolysed in a given time by a fixed amount of invertase decreased as the viscosity of the solution increased, the relation between fluidity and amount of hydrolysis being nearly linear. Since the viscosity of concentrated sucrose solutions is an exponential and not a linear function of the sugar content, it appears that the results of Colin and Chaudun are not in agreement with those of Nelson and Schubert.

The experiments of Colin and Chaudun were carried out at 23°, a temperature considerably lower than that of the critical inactivation of invertase. The optimum temperature for hydrolysis by invertase is generally taken to be 50–55° [*cf.* Oppenheimer and Kuhn, 1927; Waksman and Davison, 1926], but this value is not characteristic of the enzyme, being dependent on the time of the reaction. In view of the high negative temperature coefficient of viscosity, and the fact that an enzyme in the presence of its substrate is more resistant to high temperatures, it appeared probable that the apparent optimum temperature for the hydrolysis of concentrated sugar solutions by invertase would be higher than that for dilute substrate concentrations. The data obtained in the following experiments show that this is the case.

Experimental.

The invertase used was a very active preparation obtained from autolysed yeast. Its activity was tested from time to time and was found to remain constant during the period covered by the investigation. The stock enzyme solution was diluted immediately before use and the amount added to the substrate adjusted so that in all cases 100 cc. of sugar solution contained 0·4 cc. of invertase preparation. The stock enzyme solution was buffered with phosphoric acid to the extent that, when added in the above amount to sugar solutions, the resulting p_H was 4·4–4·6. No alteration in p_H was observed during the course of the hydrolysis.

To stop the hydrolysis 10 cc. of the reaction mixture were pipetted into a 100 cc. flask containing 80 cc. 0·25 % NaOH, and the solution was diluted with distilled water to 100 cc. at 15°. The mean delivery time of the pipette was taken as the time of observation. In view of the viscosity of the concentrated sugar solutions, the standard drain of the pipette was increased from 15 to 30 seconds, during which time the bulk of the sample was mixed with the alkali. The rotation of the sample was observed 15 to 30 minutes later. Readings were taken in a 400 mm. jacketed-tube round which water from a thermostat at 20° was circulated.

The amount of hydrolysis was calculated from the formula

$$\% \text{ hydrolysis} = \frac{R-d}{R-L}\,100,$$

where d is the observed rotation,
R is the initial rotation,
L is the rotation for complete inversion.

The initial rotation was determined for each concentration and temperature by adding to the sugar solutions the standard amount of invertase, the activity of which had been destroyed by diluting with NaOH. The final rotation was calculated from the relation

$$L = 0{\cdot}317\,R \text{ at } 20° \text{ [Hudson, 1910].}$$

The rotation of the invertase preparation was found to be negligible at the concentrations used.

The reaction velocity was calculated from the formula

$$k = \frac{1}{t}\log_{10}\frac{a}{a-x}.$$

In the experiments dealt with in Tables I–IV the amount of enzyme was constant, but the weight of substrate varied with the concentration. In order to calculate the activity of the enzyme in terms of the absolute weight of sugar hydrolysed in unit time, use was made of the expression:

$$\text{Invertase activity} = 10k \times \text{g. substrate.}$$

Table I. *The hydrolysis of solutions containing 70 g. sucrose/100 cc.*

T.	t (minutes)	% hydrolysis	$k \times 10^5$	
45°	56	27·6	250	
	88	39·2	247	
	143	57·0	255	
	163	60·1	246	Average 250
50°	60	36·8	332	
	87	49·2	338	
	137	65·9	341	
	178	75·0	338	Average 337
57°	44	39·2	491	
	73	56·2	491	
	112	72·2	496	
	144	79·3	496	
	204	89·4	(478)	Average 493
62·2°	19·5	24·5	626	
	41·5	45·8	643	
	83	69·3	618	
	114	79·8	610	Average 624
65°	15	20·84	(677)	
	40	48·4	719	
	75	70·8	714	
	115	84·1	695	Average 709
68·8°	15	25·2	840	
	41·5	54·9	833	
	76·5	76·1	813	
	120	88·4	(780)	Average 829
72°	17·5	31·4	935	
	41·5	58·0	908	
	81	78·7	(830)	
	119	87·9	(771)	Average 921
75°	26·5	41·4	876	
	50	57·4	743	
	83	69·3	618	
	128	78·8	526	

Table II. *Hydrolysis of solutions containing 55 g. sucrose/100 cc.*

T.	t (minutes)	% hydrolysis	$k \times 10^5$	
38°	54·7	35·2	344	
	91	53·5	365	
	128	66·4	370	
	163·7	75·6	374	
	225	86·0	379	Average 366
45°	25·2	25·1	(495)	
	60	51·7	527	
	88	66·5	540	
	117	76·7	541	
	165·5	86·5	526	Average 533
57°	30·7	47·0	898	
	56·3	70·2	933	
	80	81·7	922	
	107·5	89·2	898	Average 913
65°	20·5	44·3	1240	
	40	68·6	1260	
	71	87·8	1280	
	100	93·6	1190	Average 1240

Table III. *Hydrolysis of solution containing 40 g. sucrose/100 cc.*

T.	t (minutes)	% hydrolysis	$k \times 10^5$	
57°	18	49·5	1650	
	26·3	63·4	1660	
	25·7	75·0	1690	
	51·75	86·6	1680	Average 1680

Table IV. *Hydrolysis of solution containing 70 g. sucrose/100 g. solution (94·5 g./100 cc.).*

Digestion mixture 70 g. sucrose, 29·6 cc. water, 0·4 cc. invertase solution.

T.	t (minutes)	% hydrolysis	$k \times 10^5$	
57°	42	12·8	142	
	90·5	25·5	141	
	169	42·1	140·5	
	296	58·5	(130)	Average 141
65°	20	10·1	231	
	54	25·6	238	
	105	43·4	235	
	175	57·2	(211)	
	277	70·4	(191)	Average 235
70°	28·5	18·9	319	
	65·5	36·9	306	
	117·5	53·4	282	
	203	69·1	252	
	308	79·2	222	

The bracketed values for k were not used in estimating the mean value.

Table V.

T.	Concentration g./100 cc.	$10k$	$10k \times$ g. substrate
57°	40	0·167	6·68
	55	0·0913	5·02
	70	0·0493	3·45
	94·5	0·0141	0·99
65°	55	0·124	6·82
	70	0·0709	4·96
	94·5	0·0235	1·64

Fig. 1 shows that the relation between invertase activity and substrate concentration is linear.

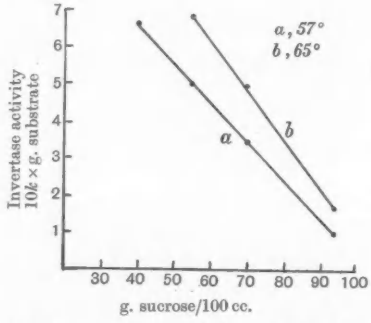


Fig. 1. Relation between invertase activity and substrate concentration.

The temperature coefficient.

From the results given in Table I it appears that for a digestion period of a few hours the apparent optimum temperature for the hydrolysis of 70 % sucrose in the presence of invertase is about 70°.

Experiments on the hydrolysis of 10 % sucrose by invertase at the same p_H showed that at 65° the value for k decreased very rapidly, while at 68° the hydrolysis ceased after 20 minutes owing to the inactivation of the enzyme.

The temperature coefficient of the reaction is also greater for concentrated, than that found by Nelson and Bloomfield [1924] for dilute, solutions. Calculation of the values of the critical increment in calories per gram-molecule gave the following results:

Table VI.

$$E = R\frac{T_1 - T_2}{T_2 - T_1}\log_e\frac{k_2}{k_1}.$$

Conc. of sugar	$T°$ (abs.)	$k \times 10^5$	Temp. range ° C.	Mean temp. $t°$ C.	E (found)	E (calc.)
70 %	318	250	45 –50	47·5	12,300	12,500
	323	337	45 –57	51	11,900	11,900
	330	493	50 –57	53·5	11,600	11,500
	335·2	624	57 –65	61	10,100	10,200
	338	709	57 –68·8	62·9	9,900	9,900
	341·8	829	62·2–72	67·1	9,200	9,200
	345	921	65·2–72	68·6	8,900	9,000
55 %	311	366	38 –45	41·5	10,600	10,600
	318	533	45 –57	51	9,400	9,600
	330	913	57 –65	61	8,500	8,500
	338	1240	—	—	—	—
94·5 %	330	141	57 –65	61	14,200	—
	338	235	—	—	—	—

The experimental values for E are plotted against the mean temperature in Fig. 2 and are in agreement with the equations

$$E = 20{,}500 - 168t \text{ for } 70\ \%\ \text{sucrose},$$
$$E = 15{,}100 - 108t \text{ for } 55\ \%\ \text{,,}\ .$$

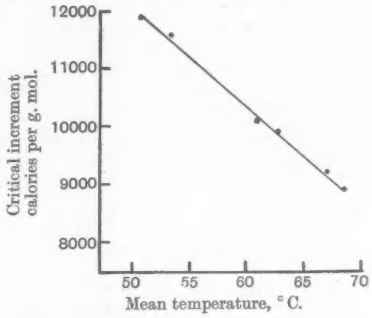


Fig. 2. Variation of the critical increment with temperature. Sucrose concentration 70 g./100 cc.

Nelson and Bloomfield [1924] combined the data in the literature for the hydrolysis of dilute sugar solution by invertase and found that the observed values could be calculated from the equation

$$E = 12{,}300 - 117t.$$

In order to ensure that the divergence between the values for E for the different substrate concentrations was not due to the enzyme preparation, the rate of hydrolysis of 10 % sucrose by invertase at p_H 4·6 was determined at 38° and 56·5°. The values of k for the unimolecular reaction were found to increase regularly as the hydrolysis proceeded and a different method was used to calculate the relative rates of hydrolysis at the two temperatures. The time t_z required by the reaction mixture to reach zero rotation (75·9 % hydrolysis) was found to be 61 and 32 minutes at 38° and 56·5° respectively. Using the reciprocals of these values to denote the relative velocities, the value of E was calculated to be 7100: the corresponding figure calculated from the equation of Nelson and Bloomfield for a mean temperature of 47·5° was 6800.

Viscosity and rate of hydrolysis.

In order to test the statement of Colin and Chaudun that the amount of hydrolysis by invertase of concentrated sucrose solutions is proportional to the fluidity of the solution, the viscosities of the solutions used were calculated. For this purpose Orth's equation [Browne, 1912] relating the viscosity of concentrated sugar solutions to the temperature and concentration, was used:

$$\log_e(\log_e \eta) = \log_e(\log_e A) + x \log_e B + t \log_e C,$$

where η is the viscosity,

x is the concentration in g./100 g. solution,

t is the centigrade temperature, and

A, B and C are constants.

Applying viscosity data from Browne [1912] and from Landolt-Börnstein [1923] the equation was simplified to the following:

$$\log_e(\log_e \eta) = 3{\cdot}619 + 0{\cdot}07798x - 0{\cdot}02260t.$$

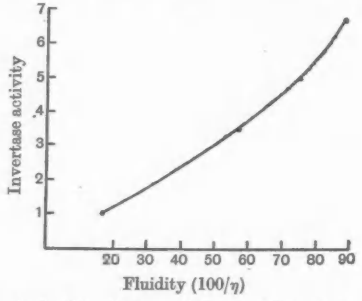


Fig. 3. Relation between the fluidity of sugar solutions and invertase activity. Temperature 57°.

In Fig. 3 the fluidity ($100/\eta$) of sucrose solutions at 57° is plotted against the invertase activity (Table VI) for the corresponding concentration. The linear relationship found by Colin and Chaudun is not observed.

Discussion.

According to Euler and Laurin [1919] the critical inactivation temperature for invertase is 59°. The observations described above lead to the conclusion that invertase resists exposure to much higher temperatures when in the presence of a large excess of substrate. The increased rate of hydrolysis observed at temperatures above that of critical inactivation is not merely an increase in the initial velocity, since the observations covered a large portion of the range of inversion. In this connection it should be noted that the course of the hydrolysis by invertase of 70 % sucrose follows that of a unimolecular reaction much more closely than does the hydrolysis of 10 % sucrose, although deviations from the mass law might be expected with the more concentrated solutions.

The larger values of the temperature coefficient of the hydrolysis of concentrated, compared with that for dilute, solutions cannot be ascribed to the increased rate of hydrolysis due to decrease in viscosity with rise in temperature, since this would not account for the greater resistance to temperature of the enzyme in presence of 70 % sugar. A concentration of 10 % sucrose is greater than that required to produce the maximum initial velocity of hydrolysis by invertase [*cf.* Kuhn, 1923], and presumably the enzyme is already saturated with substrate at this concentration. By applying the conception of Willstätter that an enzyme consists of an active substance stabilised by the presence of a colloidal carrier, the resistance of the enzyme-complex to temperature in the presence of sugar syrup may be explained as due to the increased stability of the carrier in this medium.

In Figs. 1 and 3 it is shown that the decrease in the rate of hydrolysis with increasing sucrose concentration is more closely related to the decrease in water content than to the increase in viscosity. This is in agreement with the results of Ingersoll [1926] who found that the initial velocity of sucrose hydrolysis by invertase at 25° did not vary with the increase in viscosity due to increase in sugar concentration in the manner described by Colin and Chaudun. Ingersoll also showed that the initial velocity was not decreased by the addition of gelatin in amounts sufficient to produce a considerable increase in viscosity. It may be concluded, therefore, that the viscosity of the medium is not the factor responsible for the observed variations in the catalytic activity of invertase.

The values calculated for the critical increment are subject to a possible correction for the variation of the optimum p_H with temperature and substrate concentration. Nelson and Bloomfield, however, have shown that the small shift in optimum p_H with temperature is towards the alkaline side. This means that the actual estimations were carried out slightly on the acid side of the

optimum. Since Nelson and Bloomfield have shown that the value of the critical increment is smaller at acidities greater than the optimum, it follows that any correction would increase the value of E for the concentrated solutions, and so increase the difference between the values for the dilute and concentrated substrate.

SUMMARY.

1. The rate of hydrolysis by invertase of solutions containing 40 % or more of sucrose decreases as the substrate concentration increases, the relation between substrate concentration and invertase activity being linear.

2. The optimum temperature for the hydrolysis of 70 % sucrose in the presence of invertase is 65–70°.

3. The relation between the critical increment in calories per gram-molecule and the centigrade temperature for the hydrolysis of 70 % sucrose by invertase can be expressed by the relation

$$E = 20{,}500 - 168t.$$

REFERENCES.

Browne (1912). Handbook of sugar analysis, p. 310. (Chapman and Hall, London.)
Colin and Chaudun (1922). *Bull. Soc. Chim. Biol.* **4**, 272.
Euler and Laurin (1919). *Z. physiol. Chem.* **108**, 64.
Hudson (1910). *J. Ind. Eng. Chem.* **2**, 143.
Ingersoll (1926). *Bull. Soc. Chim. Biol.* **8**, 264.
Kuhn (1923). *Z. physiol. Chem.* **125**, 28.
Landolt-Börnstein (1923). Physikalisch-Chemische Tabellen, **1**, 139. (Springer, Berlin.)
Nelson and Bloomfield (1924). *J. Amer. Chem. Soc.* **46**, 1025.
—— and Schubert (1928). *J. Amer. Chem. Soc.* **50**, 2188.
Oppenheimer and Kuhn (1927). Lehrbuch der Enzyme, p. 257. (Thieme, Leipzig.)
Waksman and Davison (1926). Enzymes, p. 179. (Baillière, Tindall and Cox, London.)

CCVI. SYNTHESIS OF *i*-β-HYDROXYGLUTAMIC ACID.

By CHARLES ROBERT HARINGTON
and SYDNEY STEWART RANDALL.

WITH A NOTE ON THE DISSOCIATION CONSTANTS.

By George Maxwell Richardson.

From the Department of Pathological Chemistry, University College Hospital Medical School, and the Biochemistry Department, Imperial College of Science and Technology, South Kensington.

(*Received October 9th, 1931.*)

The synthesis of β-hydroxyglutamic acid described by Dakin [1919] leaves much to be desired in the matter of the yields obtainable. Since the isolation of this amino-acid from natural sources also offers very considerable difficulties, the compound has not been available in sufficient amount to permit of an extended examination of its chemical and physiological properties.

According to Dakin [1918] β-hydroxyglutamic acid occurs in caseinogen to the extent of about 10 %. In view therefore of its importance as a constituent of protein and still more owing to the recent claim of Dakin, West and Howe [1930] to have isolated from liver a compound, active in curing pernicious anaemia, which yields on hydrolysis one molecule of hydroxyproline and one of β-hydroxyglutamic acid, the latter compound is of considerable biological interest and it was thought to be desirable to re-investigate the possibilities of its synthesis by a more practicable method.

Among the numerous possible syntheses explored by Dakin [1919] the simplest and most attractive from the theoretical point of view consisted in the reduction of the ethyl α-*iso*nitrosoacetonedicarboxylate of Pechmann [1891] which is an easily accessible substance. Dakin's own attempt to effect this reduction, using sodium amalgam under a variety of conditions met with failure, owing probably, as he himself suggests, to secondary reactions on the part of the aminoketo-ester which would be formed during the first stage of the reduction; nor could any more satisfactory results be obtained by the reduction of ethyl α-*iso*nitroso-β-ethoxyglutaconate.

It occurred to us that catalytic methods of reduction might find an application in this problem. It is true that the catalytic reduction of the *iso*nitroso- to the amino-group and still more that of the carbonyl to the secondary alcoholic group have, until recently, been regarded as difficult of execution. The activating effect of small amounts of iron on the catalytic reduction of carbonyl com-

pounds observed by Carothers and Adams [1923] and by Faillebin [1923] has, however, largely removed the difficulties in this case, and it has further been shown by Hartung and Munch [1929] that the reduction of an *iso*nitroso-compound to the corresponding primary amine is favoured by the presence of excess of hydrogen chloride in the reaction mixture. The latter authors have indeed achieved the direct reduction of several α-*iso*nitrosoketones of mixed aliphatic-aromatic type to the corresponding amino-alcohols, the reaction being carried out in alcoholic solution containing three molecular equivalents of hydrogen chloride and the catalyst employed being palladinised charcoal. It is to be noted that Hartung *et al.* [1930] met with exceptional difficulties in the case of *iso*nitrosoacetophenone, which was readily reduced to the amino-ketone but could be reduced to the alcohol only after substitution of water for alcohol as the solvent and introduction of fresh catalyst.

When the reduction of ethyl α-*iso*nitrosoacetonedicarboxylate was attempted by the method of Hartung and Munch [1929] it was found that hydrogen was rapidly absorbed up to 2 mols., *i.e.* in amount corresponding with the reduction of the ester to ethyl α-aminoacetonedicarboxylate. That the latter was indeed the product of the reaction at the stage under discussion was proved as follows: when boiled with 20 % hydrochloric acid it lost alcohol and carbon dioxide and the residue, when treated with alkali and mercuric chloride and steam-distilled, gave a satisfactory yield of 2 : 5-dimethylpyrazine; furthermore the partial reduction product gave, when heated with *p*-nitrophenylhydrazine in excess of dilute sulphuric acid, the characteristic *p*-nitrophenylosazone of methylglyoxal.

The reduction could not be carried beyond this stage at a reasonable rate under the conditions already described. If, however, the solution were evaporated and the residue re-dissolved in water, the remaining stage of the reduction could be accomplished in presence of the original catalyst activated by the addition of chloroplatinic acid and a trace of iron. Even so, the second stage (reduction of the carbonyl group) was much slower than the first (reduction of the *iso*nitroso-group). When the uptake of hydrogen finally ceased the primary amino-nitrogen in the solution represented only about 40 % of the total nitrogen. It was apparent therefore that some ring closure had occurred, probably to give a pyrrolidonecarboxylic acid derivative; in any case the whole of the nitrogen could be converted into the amino-condition by boiling with 20 % hydrochloric acid, a process which simultaneously removed the ester groups.

At this point in the earlier experiments hydrochloric acid was removed as far as possible by evaporation of the solution under diminished pressure and the amino-acid was isolated from the residue through its silver salt as described by Dakin [1918]. Subsequently, however, the favourable properties of the hydrochloride (see below) have been utilised to obtain the bulk of the product directly as a crystalline derivative. In either case the total yield of the amino-acid amounted to about 60 % of the theoretical calculated on the ethyl α-*iso*nitrosoacetonedicarboxylate employed.

The free β-hydroxyglutamic acid obtained in the earlier experiments, being finally isolated by precipitation of its concentrated aqueous solution with alcohol, formed an amorphous powder apparently similar to the product obtained by Dakin. On one occasion, however, the mother-liquor, which contained more water than usual, deposited a little of the amino-acid after long standing in the form of crystalline crusts, and since that time no difficulty has been experienced in obtaining the acid in a crystalline condition from concentrated aqueous solution.

Since β-hydroxyglutamic acid contains two independent asymmetric carbon atoms it is evident that the synthetic product may consist of either one of two racemates or of a mixture. We have at present no satisfactory evidence to offer as a basis for a decision between these alternatives. Although we have at times observed the separation of the acid in crystals of apparently different forms, these forms have not shown any marked differences of solubility, nor have we been able to standardise the conditions of their separation. The more usual type of crystals obtained consists of stout prisms containing water of crystallisation which show no tendency to separate into different components when fractionally crystallised.

The constitution of the synthetic acid was proved by analysis of the acid itself, of its hydrochloride and of various metallic salts; also by the formation of malic semialdehyde as the result of oxidising the acid with 1 mol. of chloramine-*T*. Unfortunately we have so far been unable to isolate the amino-acid from natural sources, so that no direct comparison between the natural and synthetic products has been possible. It is indeed unlikely that any such comparison would be useful for purposes of identification, since for stereochemical reasons the physical properties of the naturally occurring active acid may well be profoundly different from those of the synthetic material. That some such marked difference does indeed exist is indicated by certain contrasts between the behaviour of our product and that of the amino-acid isolated by Dakin.

i-β-Hydroxyglutamic acid crystallises from concentrated aqueous solutions in well-formed prisms containing three molecules of water of crystallisation; it is extremely soluble in hot water but only moderately so in cold. Under certain circumstances, *e.g.* crystallisation from very concentrated solutions at a high temperature, the acid may separate in the anhydrous condition. The hydrated acid melts at 75°, re-solidifies and finally decomposes at 185°; the anhydrous compound melts sharply with decomposition at 198°. This is in marked contrast with the behaviour described by Dakin [1918] whose product had no sharp melting-point, but even at 105° lost water with ring closure to a hydroxypyrrolidonecarboxylic acid. No such ring closure takes place when the dry synthetic acid is heated at 110° even in a vacuum over phosphoric oxide. On the other hand, ring closure does occur when the amino-acid is boiled in aqueous solution, and at p_H 4 the process is considerably more rapid than with glutamic acid under similar conditions.

The synthetic amino-acid yields a well-formed hydrochloride somewhat

similar in character to glutamic acid hydrochloride although naturally more soluble than the latter. No mention of such a derivative is made by Dakin either in connection with natural or synthetic β-hydroxyglutamic acid. We now suspect, however, from some observations which we have made ourselves on the character of the crude precipitate of glutamic acid hydrochloride obtained from caseinogen in the usual manner, that unsuspected losses as the hydrochloride may account in part at least for the many failures to isolate β-hydroxyglutamic acid. In this connection the claim of Engeland [1922, footnote p. 136], based on methylation experiments, that a commercial preparation of glutamic acid hydrochloride contained as much as 50 % of β-hydroxyglutamic acid is of interest.

Finally as to colour reactions. Dakin described two principal colour reactions as characteristic for β-hydroxyglutamic acid, (*a*) a red colour with sodium diazobenzenesulphonate in presence of excess of sodium hydroxide, which deepened in intensity on warming, and (*b*) a green fluorescence on warming a solution of the acid with excess of sulphuric acid and a trace of β-naphthol. The pure synthetic amino-acid gives no colour whatever with sodium diazobenzenesulphonate and sodium hydroxide in the cold; on boiling the solution a bluish-red colour with no trace of yellow tinge is obtained[1]. The reaction is not, however, very sensitive. The green fluorescence with β-naphthol and sulphuric acid is given by the synthetic amino-acid but again not with great intensity. In any case we do not regard either of these reactions as of great value. Traces of histidine entirely obscure the result of the former test, and there appear to be substances present in protein hydrolysates which give the β-naphthol-sulphuric acid reaction with even greater intensity than β-hydroxyglutamic acid itself.

It appears therefore that marked discrepancies exist between the behaviour of our synthetic product and that of the acid isolated by Dakin from natural sources. Many of these discrepancies could doubtless be explained on stereochemical grounds and it is therefore better not to discuss them further until the resolution of the synthetic acid has been effected and until a reliable method for the isolation of the natural acid has been devised. The first of these problems we hope to take up in the near future; with the second we have been occupied for some time without success. We have repeatedly followed with meticulous care the procedure recommended by Dakin [1918] but have invariably failed to obtain the acid in the manner which he describes. The fraction which should contain β-hydroxyglutamic acid has always consisted of a small amount of gummy material more or less heavily contaminated with glutamic and aspartic acids. That β-hydroxyglutamic acid does indeed occur among the products of hydrolysis of caseinogen we do not for a moment doubt; unequivocal proof of this is afforded by the fact that certain fractions of the

[1] In a private communication to one of us Dr H. D. Dakin agrees that, contrary to his original statement, no colour should be given with sodium diazobenzenesulphonate and sodium hydroxide in the cold.

hydrolytic products yield not inconsiderable amounts of malic semialdehyde when oxidised with chloramine-*T*. We are, however, forced to the conclusion that the separation of β-hydroxyglutamic acid from the other dicarboxylic amino-acids is not by any means so straightforward a process as the original description by Dakin [1918] might lead one to suppose. The mere separation of a synthetic mixture of glutamic and β-hydroxyglutamic acids into its components is a task of considerable difficulty, although our experiments on this point, detailed description of which is reserved for a later communication, have afforded indications of possible lines of attack on the major problem. In the meantime the synthesis of the inactive amino-acid and its properties together with those of some of its simple derivatives are described below.

One of us (C.R.H.) has, during the progress of this work, enjoyed the benefit of consultation by correspondence with Dr H. D. Dakin, concerning the various points which have arisen.

Experimental.

Preparation of ethyl α-iso*nitrosoacetonedicarboxylate.*

The following method has been found more convenient for the preparation of the above compound in quantity than that described by Pechmann [1891].

Ethyl acetonedicarboxylate (20 g.) is dissolved in ether (200 cc.) in a 500 cc. round-bottom flask under a reflux condenser. Hydrogen chloride is passed into the solution at the rate of 2–3 bubbles per second. A solution of ethyl nitrite [*cf.* Thiele and Eichwede, 1900, footnote p. 366] (8·4 cc.) in ether (50 cc.) is added in 10 cc. portions through the condenser. Each addition of ethyl nitrite causes a yellowish-brown coloration and a slight rise of temperature; a fresh quantity of ethyl nitrite is only added when the colour produced by the preceding addition has been discharged. After the final addition of ethyl nitrite, passage of hydrogen chloride is continued for 15 minutes, and the mixture is then set aside overnight. Next day the bulk of the ether is distilled off, and the residue is twice shaken out with excess of dilute sodium carbonate solution. The latter is then acidified with hydrochloric acid and extracted four times with ether. The combined ethereal extracts are dried over calcium chloride and evaporated, finally in a vacuum desiccator over sulphuric acid. The yield of pure ester is almost quantitative.

Reduction of ethyl α-iso*nitrosoacetonedicarboxylate.*

The catalyst was prepared by dissolving 1 g. of palladium chloride in 100 cc. of water with the addition of 1 cc. of concentrated hydrochloric acid, adding 6 g. of "super-norite" charcoal and shaking in an atmosphere of hydrogen until reduction was complete. The product was filtered off, washed with water until free from chloride and dried in a vacuum desiccator. Ethyl α-*iso*nitrosoacetonedicarboxylate (10 g.) was dissolved in absolute alcohol (100 cc.) containing hydrogen chloride (5 g.); 3 g. of the palladium-charcoal were added and

the mixture was shaken in an atmosphere of hydrogen. Absorption of the latter occurred rapidly and continued at a moderate rate until 2 mols. had been taken up when the process became extremely slow; in various experiments this first stage occupied 1·5 to 2·5 hours.

When the absorption had practically ceased the catalyst was filtered off and washed with alcohol; the filtrate and washings were freed from alcohol by evaporation under diminished pressure and the residue was taken up in water (100 cc.). The original catalyst was returned to the solution and in addition there were added 5 cc. of a solution containing 10 % of chloroplatinic acid and 0·5 % of ferric chloride. Hydrogenation was then begun again and allowed to proceed until the remaining 1 mol. had been absorbed. When this had occurred (after 6–8 hours) the solution was filtered. The catalyst was extracted with boiling water and the combined filtrates were evaporated to 100 cc. under diminished pressure.

i-β-Hydroxyglutamic acid.

The solution obtained as described above should theoretically contain ethyl β-hydroxyglutamate. It was found, however, that only about 40 % of the total nitrogen was in the amino condition. Concentrated hydrochloric acid (100 cc.) was therefore added and the whole was boiled under a reflux for 1·5 hours. After this treatment the amino-nitrogen and total nitrogen were identical. Subsequent treatment followed two alternative lines.

(*a*) The solution was evaporated under diminished pressure, the process being repeated several times after addition of small amounts of water. The residue was taken up in water and treated with 20 % silver nitrate in amount just sufficient to precipitate the remaining hydrochloric acid. The filtrate from the silver chloride was made faintly alkaline to litmus and further silver nitrate solution was cautiously added. The precipitate produced by the first few drops immediately blackened, owing doubtless to the persistence of some reducing material. The addition of silver nitrate solution was carefully continued until a transient white precipitate was produced; at this stage the solution was set aside overnight. Next day the black precipitate was removed by filtration and the amino-acid was completely precipitated from the filtrate by alternate addition of 20 % silver nitrate and 2*N* sodium hydroxide. The silver salt was filtered off, thoroughly washed with water, and decomposed with hydrogen sulphide. The filtrate from the silver sulphide was concentrated to a thin syrup under diminished pressure and poured into excess of alcohol. The precipitate, at first sticky, gradually hardened and was ultimately obtained as a friable powder; it was dried in a vacuum desiccator over phosphoric oxide.

(*b*) After the properties of the well-characterised hydrochloride had been discovered it was found more convenient to evaporate the hydrochloric acid solution resulting from hydrolysis of the reduction product to a thin syrup under diminished pressure and to saturate the residue at 0° with hydrogen

chloride. After standing overnight in the ice-chest the hydrochloride was filtered off and the remainder of the amino-acid was isolated from the mother-liquor by the method described above. The free amino-acid was obtained in the crystalline condition by addition of 1 equivalent of sodium hydroxide to the concentrated aqueous solution of the hydrochloride, or by dissolving the amorphous preparation obtained as described above in a small amount of hot water and allowing the solution to stand. Occasionally, if the solution be very concentrated and warm when crystallisation commences, the acid separates in the anhydrous condition in stout needles or narrow rhombic prisms. More usually, however, it is obtained as stout prisms which may reach a considerable size and contain $3H_2O$; the latter is lost in a vacuum over phosphoric oxide.

Analysis. 0·1554 g. of the air-dried acid lost 0·0378 g. *in vacuo* at 50° over phosphoric oxide. Found: loss of weight 24·3 %; calculated for $C_5H_9O_5N$, $3H_2O$, loss of weight 24·9 %.

The anhydrous acid gave: C, 36·5, 36·8 %; H, 5·6, 5·5 %; N, 8·5, 8·6 %; amino-N, 8·7 %. $C_5H_9O_5N$ requires: C, 36·8 %; H, 5·5 %; N, 8·6 %.

0·2082 g. required for neutralisation to phenolphthalein 12·48 cc. of 0·099 *N* sodium hydroxide. The acid therefore titrates as a monobasic acid of mol. wt. 168 as against the calculated value of 163.

Behaviour on heating. When heated in a capillary tube the air-dried acid melts partially at about 75° and then re-solidifies; at 102–103° obvious loss of water occurs and the whole finally decomposes at 185°. The anhydrous acid melts sharply with vigorous effervescence at 198°. No apparent change occurs when the dry amino-acid is heated at 110° either in the air or in a vacuum over phosphoric oxide. That ring closure can be brought about with moderate ease is shown, however, by the following experiment. 0·5 g. of the acid was dissolved in 5 cc. of water and the solution was boiled under reflux for 7 hours, by which time the ninhydrin reaction had become very faint. Analysis indicated more than 80 % loss of amino-nitrogen. The solution was evaporated to a syrup on the water-bath and the residue was extracted with alcohol. The extract was filtered from unchanged amino-acid and evaporated again to a syrup which slowly crystallised. The product had N, 9·4 % (calculated for $C_5H_7O_4N$; N, 9·65 %) and yielded only a trace of nitrogen in the Van Slyke apparatus. A comparison between the rates of ring closure of glutamic and β-hydroxyglutamic acids was kindly carried out by Prof. A. C. Chibnall who obtained the following figures (the acids were boiled in aqueous solution at p_H 4).

	% of total N as amino-N after		
	1 hr.	2 hrs.	3 hrs.
Glutamic acid	96·9	93·0	84·3
β-Hydroxyglutamic acid	81·2	75·5	62·7

Reactions. The acid, after neutralisation to litmus, gives an intense ninhydrin reaction. With sodium diazobenzenesulphonate and excess of sodium hydroxide it gives no colour in the cold; on boiling a bluish-red colour develops.

When an aqueous solution of the acid is treated with rather more than its own volume of sulphuric acid and the mixture is warmed with a trace of β-naphthol a yellowish solution with a green fluorescence results. The vapours evolved on fusing the acid with zinc dust give an intense pine splinter reaction for pyrrole.

Oxidation with chloramine-T. 0·815 g. of the acid in 5 cc. of water was neutralised with *N* sodium hydroxide (5 cc.) and treated with a solution of chloramine-*T* (1·44 g.) in water (5 cc.). Almost immediate precipitation of toluenesulphonamide occurred. After 30 minutes the reaction was completed by gentle warming, the solution was chilled and the toluenesulphonamide filtered off. The filtrate was treated with a hot solution of *p*-nitrophenylhydrazine (2 g.) in glacial acetic acid and transferred to the steam-bath. Precipitation of the osazone began at once; after 45 minutes the precipitate was filtered off, washed with warm alcohol and dried; it weighed 0·5 g. After recrystallisation from nitrobenzene it formed brownish-red prismatic needles, M.P. 291° (uncorr.). (Found: N, 22·6 %. $C_{16}H_{14}O_6N_6$ requires: N, 22·6 %.)

Derivatives of i-*β-hydroxyglutamic acid.*

Hydrochloride. This was prepared as described above. It forms colourless prismatic needles, readily soluble in water, sparingly so in concentrated hydrochloric acid. It has M.P. 187° (decomp.). (Found: C, 29·8, 30·0 %; H, 5·0, 5·1 %; N, 7·1, 7·0 %; amino-N, 7·3 %; Cl, 17·7 %. $C_5H_{10}O_5NCl$ requires: C, 30·0 %; H, 5·0 %; N, 7·0 %; Cl, 17·8 %.)

Ethyl ester hydrochloride. β-Hydroxyglutamic acid hydrochloride (7 g.) was suspended in alcohol (70 cc.) and the mixture was saturated with hydrogen chloride. The resulting clear solution was boiled under reflux for 3 hours and then concentrated under diminished pressure. During this process the greater part of the ester hydrochloride crystallised out. This was filtered off (6 g.) and recrystallised from alcohol. M.P. 168·5°. (Found: N, 5·2 %. $C_9H_{18}O_5NCl$ requires: N, 5·5 %.)

Silver salt. Prepared by adding silver nitrate (2 mols.) to a solution of the disodium salt of β-hydroxyglutamic acid (1 mol.) filtering, washing and drying in a vacuum desiccator in the dark. (Found: N, 3·6 %. $C_5H_7O_5NAg_2$ requires: N, 3·7 %.)

The normal *copper* salt had N, 5·9 %, Cu, 28·0 % (calc.: N, 6·2 %, Cu, 28·3 %) and the *calcium hydrogen* salt had Ca, 11·5 % (calc.: Ca, 11·0 %). These salts were prepared according to Dakin [1918] and resembled the products described by him.

Identification of intermediate product in the reduction of ethyl α-isonitrosoacetonedicarboxylate.

(*a*) Ethyl α-*iso*nitrosoacetonedicarboxylate (5 g.) was subjected to the first stage of the reduction in the manner described above. The residue obtained by evaporation of the alcoholic solution was dissolved in 20 % hydrochloric acid (50 cc.) and boiled under reflux. Decarboxylation set in rapidly and was

complete in 45 minutes. The solution was evaporated under diminished pressure, the evaporation being several times repeated after addition of small amounts of water. The residue was taken up in water (35 cc.) and the solution was added with cooling to 80 cc. of 33 % potassium hydroxide; 10 g. of mercuric chloride dissolved in cold water (100 cc.) were then added and the whole was immediately steam-distilled. After 75 cc. had passed over the base had practically ceased distilling. The distillate was treated with a solution of mercuric chloride which produced a crystalline precipitate of the mercuric chloride double salt with 2 : 5-dimethylpyrazine; after standing overnight the precipitate was collected, washed and dried in a desiccator. It amounted to 5 g. corresponding with a yield of 70·5 % of the theoretical calculated on the ester originally taken. (Found: Hg, 62·2 %. $C_6H_8N_2,2HgCl$ requires: Hg, 61·7 %.) The base recovered from the mercuric chloride salt was further identified by conversion into the *picrate*, M.P. 157°. (Found: picric acid, 68·5 %; calculated, 67·9 %), and the *chloroaurate*, M.P. 153°.

(*b*) The residue from the evaporation of the alcoholic solution of the first stage reduction product from a second quantity of 5 g. of ethyl α-*iso*nitrosoacetonedicarboxylate was treated with a solution of *p*-nitrophenylhydrazine (5 g.) in excess of hot 5 % sulphuric acid and transferred to the steam-bath. Within a few minutes the solution became filled with the characteristic scarlet needles of methylglyoxal *p*-nitrophenylosazone. After 1 hour the precipitate was filtered off, washed with alcohol and dried (1·4 g.). After recrystallisation from nitrobenzene it had M.P. 297° (decomp. uncorr.).

NOTE ON THE DISSOCIATION CONSTANTS.

By GEORGE MAXWELL RICHARDSON[1].

Application of amino-acid dissociation constants to the interpretation of protein titration curves is a recognised procedure which depends upon accurate knowledge of these constants and concise statement of the conditions of measurement. Data are now presented for crystalline preparations of β-hydroxyglutamic acid, accessible to measurement for the first time.

Bjerrum's zwitterion hypothesis, which possesses the cumulative experimental evidence of Borsook and MacFadyen [1930], Michaelis and Mizutani [1925], and Birch and Harris [1930, 1, 2] in support of its more logical interpretation of the behaviour of amino-acid solutions, is adopted throughout the text. Basic dissociation constants are calculated as constants of hydrion dissociation, after Brønsted [1923]. These generally accepted conventions,

[1] Beit Scientific Research Fellow.

presented more fully by Greenstein [1931], allow an abbreviated formulation of the acid-base exchanges as follows:

$$\overset{-\,H^+}{{}^+A} \rightleftharpoons \overset{-\,H^+}{{}^+A^-} \rightleftharpoons \overset{-\,H^+}{{}^+A^{--}} \rightleftharpoons A^{---}.$$

Decreasing magnitudes, K_1', K_2', K_3', represent the successive dissociation constants (uncorrected for activity of the various species of A) possible within this scheme. In this paper, however, calculation of the slightly different

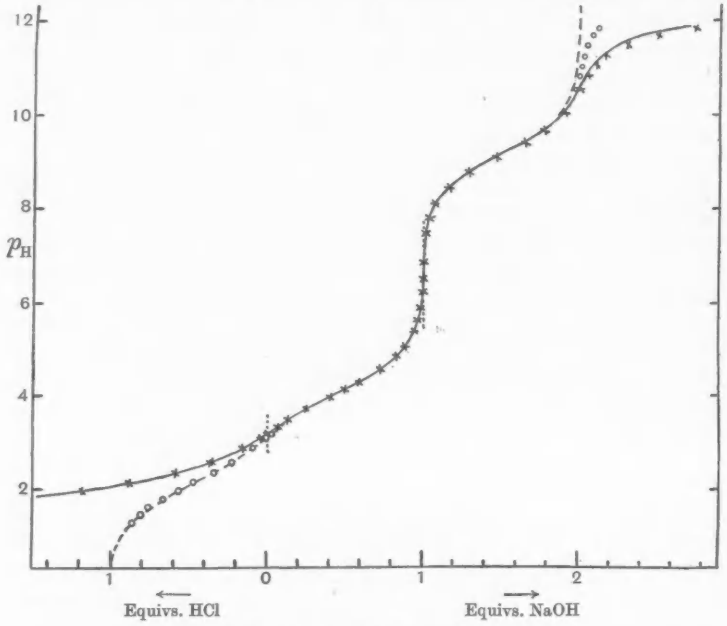


Fig. 1. Titration of 0·02 *M* β-hydroxyglutamic acid, 25°.

Calculated titre, uncorrected ——— Corrected - - - - - -
Experimental titre, uncorrected × Corrected ○

titration constants of Simms [1926], G_1', G_2', G_3', is made. It is to be emphasised that electrometric titration of any dicarboxylic amino-acid is attended by the usual difficulties of technique and calculation relating to comparatively acid solutions, and that this susceptibility to error will necessarily appear in the values of G_1'.

Experimental data are presented in Fig. 1, the curve representing calculated requirements, the points experimental findings. Table I presents a portion of these data arranged for calculating Simms's titration constants. Experimental titres are corrected throughout for the concentration of uncombined HCl or

NaOH at the stated p_H. Bracketed values represent determinations in poorly buffered regions, or values embodying heavy correction. The Sørensen logarithmic nomenclature (p_{G_1}, *etc.*) is adopted.

Table I. *Calculation of titration constants.*

25°. 0·02 *M* β-hydroxyglutamic acid, $3H_2O$. 0·1012 *M* NaOH. 0·1972 *M* HCl.

p_{G_2}' (0·1122 g. acid in 25 cc.)

NaOH cc.	p_H	μ	Correction (equivs.) for H+	+A	+A−−	α_2	p_{G_2}'
0	3·219	0·00193	0·0299	0·0704	—	0·1003	4·172
0·32	3·368	0·00262	0·0216	0·0510	—	0·1352	4·174
0·65	3·518	0·00347	0·0156	0·0366	—	0·1794	4·178
1·25	3·753	0·00531	0·0093	0·0216	—	0·2756	4·173
2·02	4·019	0·00782	0·0053	0·0119	—	0·4126	4·172
2·50	4·176	0·00936	0·0037	0·0083	—	0·5014	4·174
3·00	4·337	0·0109	0·0027	0·0057	—	0·5957	4·169
3·70	4·598	0·0131	0·0015	0·0032	—	0·7291	4·168
4·24	4·863	0·0147	0·0008	0·0017	—	0·8326	4·166
4·51	5·054	0·0155	0·0005	0·0011	0·0001	0·8845	4·170
4·81	5·381	0·0161	0·0003	0·0005	0·0002	0·9423	{4·168
5·01	5·900	0·0167	0·0001	0·0002	0·0005	0·9806	4·196
5·06	6·240	0·0168	—	0·0001	0·0011	0·9896	4·262}
5·108 (=first equiv.)							Mean 4·172

p_{G_1}' (0·1089 g. in 25 cc.)

HCl cc.	p_H	p_{G_1}'
0	3·221	2·10
0·125	3·106	2·09
0·40	2·886	2·09
0·90	2·595	2·10
0·93	2·582	2·10
1·49	2·357	2·09
2·25	2·149	2·11
3·01	1·989	{2·11
4·25	1·803	2·10
6·01	1·634	2·14
9·76	1·418	2·06
13·60	1·299	2·10}
		Mean 2·10

p_{G_3}' (0·1122 g. in 25 cc.)

NaOH cc.	p_H	p_{G_3}'
5·12	6·881	9·20
5·20	7·485	9·20
5·30	7·817	9·23
5·49	8·126	9·22
5·92	8·474	9·21
6·60	8·801	9·20
7·49	9·119	9·20
8·41	9·423	9·19
9·10	9·686	9·18
9·75	10·057	9·19
10·25	10·553	{9·20
10·51	10·860	9·19}
11·10	11·262	—
11·76	11·497	—
12·77	11·700	—
14·02	11·859	—
		Mean 9·20

In discussion of the data various points arise. Firstly, the table provides an interesting comparison between the very constant p_{G_2}', values, where ionic strength and H^+ concentration are both comparatively low, and the erratic p_{G_1}', or the decreasing p_{G_3}' values, where a large correction for free H^+, or a rapidly increasing ionic strength, respectively, are involved. Secondly, certain evidence for a fourth (hydroxy) dissociation constant at about p_H 13 may be detected in Fig. 1, though the absence of any satisfactory basis for correcting for free OH^- precludes definite evaluation of the constant. An attempt to eliminate the effect by larger OH^- correction disturbs the terminal p_{G_3}' values and fails to give good alignment of the end-point. Thirdly, preliminary titration of a preparation, dried at 50° over P_2O_5, revealed that titratable

groups about p_H 9 exceeded the more acid titratable groups by about 4 %. A further recrystallisation, however, reduced the extent of the divergence to about 2 %, approaching the limits of possible experimental error. The interfering $p_{G'}$ lay between 9·0 and 9·4. As neither analysis nor method of preparation offers an easy explanation for a defect of —COOH as against $—NH_2$ groups, the observation is merely recorded. Fourthly, it may be noted that an air-dried sample of the β-hydroxyglutamic acid containing $3H_2O$ was found to titrate with respect to its acid groups as 75 % hydroxyglutamic acid by weight (theory 75·1 %). The anhydrous preparation titrated as 99 % acid.

Table II. *Dissociation constants at 25°.*

	Mol. conc.	—COOH	—COOH	$—NH_2$	Diff. (pK_{NH_2}' – pK_{NH_2}')	Authors
Glutamic acid	—	2·15	4·22	9·60	—	Various [*vide* Kirk and Schmidt, 1929]
β-Hydroxyglutamic acid	—	2·32	4·23	9·56	0·04	[Kirk and Schmidt, 1929]
β-Hydroxyglutamic acid	0·02	2·09	4·18	9·20	0·40	This paper
β-Asparagine	0·02	2·02	—	8·80	—	[Chibnall and Cannan, 1930]
β-Hydroxyasparagine	0·01	2·12	—	8·26	0·54	,, ,, ,,
Aspartic acid	0·02	2·05	3·76	9·85	—	,, ,, ,,
Hydroxyaspartic acid	0·02	1·95	3·47	9·03	0·82	,, ,, ,,

The comparison of the constants here determined with those of Kirk and Schmidt [1929] reveals definite discrepancy in the case of the third (amino) dissociation constant. It is believed, however, that the synthetic origin and purity of our preparation, and the satisfactory mathematical analysis accorded by the data are sufficient evidence for the greater reliability of the present constants. This belief is further supported by the comparisons of Table II, in which our data are compared with those of Kirk and Schmidt, and with the data of Chibnall and Cannan [1930] for hydroxyaspartic acid and its amides. The influence of an adjacent hydroxyl group on the $p_{G'}$ of the amino-group is to be noted. In particular, the glutamic acids should be compared with the β-asparagines, where there is likewise an α-amino group, a β-hydroxy group, and an almost non-polar γ-group.

Finally, for general reference, the $p_{G'}$ constants are converted to the real dissociation constants by the formula of Simms [1926]. Thus, the successive values of $p_{K'}$, determined by titration of 0·02 *M* β-hydroxyglutamic acid at 25°, become 2·09, 4·18 and 9·20.

Experimental.

Titrations were conducted in the manner of Cannan and Knight [1927]. Reagents and apparatus were prepared and standardised with the usual precautions. Temperature was maintained closely at 25°. The half-cell adopted was a saturated calomel electrode, standardised against 0·1 *M* HCl from a constant boiling-point stock. Junction was made at the tip of a goose-neck saturated KCl bridge of the Clark type. The H_2 electrode potential assumed for 0·1 *M* HCl was 0·0637v., junction potentials being deliberately ignored throughout.

Care was observed particularly during acid standardisation or titration, to prevent fortuitous junction potentials arising from a dead space in the tip, containing, between the KCl and the external liquid, a solution of some previous p_H. On the other hand, increase of ionic strength of the solution due to KCl introduction was minimised. Potentials were established at duplicate, freshly palladinised, gold-plated platinum wire electrodes, and measured against a Cambridge potentiometer and galvanometer. In no observation did duplicate potentials differ by more than 0·2 mv. and there was generally agreement closer than 0·1 mv. An exception arose, however, with those less pure preparations already mentioned, variations up to 0·4 mv. occurring about p_H 10.

Finally, due mention must be made of the basis upon which observed H^+ activities (p_H) were converted into H^+ or OH^- concentrations. The simplified Debye-Hückel equation, $-\log\gamma = a\sqrt{\mu}$, where a varies with the dielectric constant of the solution and the degree of solvation of the ions, was used. In these calculations, a has been accepted, empirically, as a constant, 0·245, for both H^+ and OH^-.[1]

In calculation of μ, the ionic strength, it is assumed for simplicity that a positive and negative charge on the same ion are mutually neutralised in their influence on the ionic atmosphere. Thus $^+A^-$ is disregarded and $^+A^{--}$ is regarded as a univalent ion [compare Greenstein, 1931]. On this basis, μ increases most rapidly during the reaction $^+A^{--} \rightarrow A^{--}$, which would produce just that gradual decrease in $p_{G_2'}$ values which Table I reveals. On the other hand, the full assumption is contrary to the conclusions of Borsook and MacFadyen [1930], who wittingly offer no alternative formulation for the modified participation of such ions. On the stated basis, μ is readily shown to equal the sum

$$[^+A] + [H^+] + [Na^+] + [A^{--}],$$

where, with negligible error,

$$[^+A] = [A]\frac{H^2}{(G_1+H)(G_2+H)}, \text{ and } [A^{--}] = [A]\frac{G_2}{(G_2+H)},$$

[A] representing total amino-acid concentration and H, H^+ activity. OH^- activities were derived from H^+ activities by assuming Lewis, Brighton, and Sebastian's value [1917] for p_{K_w}, 13·995, without modification for altered dielectric constant.

[1] The activity of these two ions in solutions of moderate ionic strength is shown by data of Lewis and Randall to be closely comparable, while the above value of a is of similar order to that adopted by Simms for H^+ (0·28). It differs from the decreasing value of Randall and Breckenridge [1927] for chloride solutions of increasing ionic strength. It was found that the values of these authors gave no concordant data in the calculation of $p_{G_1'}$. The critical survey of Borsook and MacFadyen [1930] is sufficient justification in this case for selecting an empirical constant.

REFERENCES.

Birch and Harris (1930, 1). *Biochem. J.* **24**, 564.
—— —— (1930, 2). *Biochem. J.* **24**, 1080.
Borsook and MacFadyen (1930). *J. Gen. Physiol.* **13**, 509.
Brønsted (1923). *Rec. Trav. Chim.* **42**, 718.
Cannan and Knight (1927). *Biochem. J.* **21**, 1384.
Carothers and Adams (1923). *J. Amer. Chem. Soc.* **45**, 1071.
Chibnall and Cannan (1930). *Biochem. J.* **24**, 945.
Dakin (1918). *Biochem. J.* **12**, 290.
—— (1919). *Biochem. J.* **13**, 398.
—— West and Howe (1930). *Proc. Soc. Exp. Biol. Med.* **28**, 2.
Engeland (1922). *Z. physiol. Chem.* **120**, 130.
Faillebin (1923). *Compt. Rend. Acad. Sci.* **177**, 1118.
Greenstein (1931). *J. Biol. Chem.* **93**, 479.
Hartung and Munch (1929). *J. Amer. Chem. Soc.* **51**, 2262.
—— Munch, Deckert and Crossley (1930). *J. Amer. Chem. Soc.* **52**, 3317.
Kirk and Schmidt (1929). *Univ. California Publ. Physiol.* **7**, 57; *J. Biol. Chem.* **81**, 237.
Lewis, Brighton and Sebastian (1917). *J. Amer. Chem. Soc.* **39**, 2245.
Michaelis and Mizutani (1925). *Z. physikal. Chem.* **116**, 135.
Pechmann (1891). *Ber. deutsch. chem. Ges.* **24**, 860.
Randall and Breckenridge (1927). *J. Amer. Chem. Soc.* **49**, 1435.
Simms (1926). *J. Amer. Chem. Soc.* **48**, 1239.
Thiele and Eichwede (1900). *Liebig's Ann.* **311**, 363.

CCVII. STUDY OF THE BLOOD-CALCIUM FOLLOWING THE INTRAVENOUS INJECTION OF CALCIUM SALTS.

By GUY DRUMMOND GREVILLE.

From the Courtauld Institute of Biochemistry, Middlesex Hospital, London.

(*Received October 10th, 1931.*)

Through the intravenous administration of calcium salts the blood suffers only a transitory increase in calcium concentration, for the blood-calcium sinks rapidly, returning usually within 2 hours of the injection to its original level. This has been observed by several workers in different animals, notably by Heubner and Rona [1919, 1] in cats, by Hetényi [1924] and Gerschmann [1930] in dogs, and by Clark [1920], Condorelli [1926, 1927] and Rothlin [1930] in rabbits. The last two authors and Taylor and Fine [1930] have commented on the remarkably large apparent loss of calcium from the blood-stream during the first 5 minutes after the injection. The object of the experiments described below was firstly to discover whether this apparent loss could be attributed in any measure to shortcomings of the analytical method, and secondly to study the kinetics of the fall of the blood-calcium.

Material.

In addition to calcium chloride, there was used in this work calcium levulinate, a very soluble calcium salt described by Conrad [1878], the value of which as an agent for calcium therapy by injection has recently been pointed out [Dodds and Greville, 1931; Bennett, 1931]. Pure calcium levulinate was kindly supplied by the Crookes Laboratories.

Adequacy of the analytical method.

In most of the researches on the behaviour of the serum-calcium following calcium administration, the calcium has been determined by direct precipitation as oxalate from diluted serum, usually by the technique of Kramer and Tisdall [1921] as modified by Clark and Collip [1925]. This much-used procedure of direct precipitation has recently been severely criticised. Incomplete calcium precipitation due to the presence of serum-proteins tends to lower the result [Van Slyke and Sendroy, 1929; Guillaumin, 1930], while at the same time entrainment of oxidisable foreign matter with the oxalate precipitate tends to raise it. Guillaumin [1930] concluded that with fresh human blood the second error preponderates. So that although direct precipitation has often been shown in animal serum to give the same results as

ashing [Loucks and Scott, 1929–30; Hendriks, 1929], it must be recognised that error may possibly accompany its use.

Experimental.

It was first shown that quite good recoveries were obtained by the Clark and Collip technique when large quantities of calcium were added to serum. To 2 cc. of horse serum in the precipitation-tubes measured volumes of standard calcium chloride solution were added. Analyses were performed in quadruplicate, the extreme error being less than 2 %. Recoveries of 98·4 % and 97·6 % were obtained with 0·577 mg. and 0·289 mg. of added calcium respectively.

The above results were obtained when serum and dilute calcium chloride solution were mixed in the precipitation-tube. If, on the other hand, a mixture was made by adding, with thorough shaking, 1 cc. of a stronger calcium solution to 49 cc. of serum in a graduated flask, 2 cc. portions of this mixture gave, when analysed by direct precipitation, low and erratic results. Thus with a mixture made as above from horse serum and calcium levulinate solution, with a total calcium content of 39·0 mg./100 cc., values ranging from 10 to 33 mg./100 cc. were obtained. Similar results were obtained with a mixture of calcium chloride solution and horse serum. The error was not due to insufficient mixing since consistent, if rather high, recoveries were obtained when the calcium was determined in the mixture following precipitation of the proteins by trichloroacetic acid. No systematic alteration occurred when determinations were made after leaving the mixture for many hours and centrifuging, or when the oxalate precipitation was allowed to proceed for 24 hours. The supernatant liquid after centrifuging the precipitate, and the washings, were observed to be cloudy; hence under these conditions the calcium oxalate is precipitated in a readily peptisable form, and variable amounts of colloidal calcium oxalate are decanted off in the supernatant fluid. It is interesting to note that Grollman [1927] found that, when calcium chloride was added to serum *in vitro* in amounts (up to a total concentration of 32 mg./100 cc.) which caused no precipitation or change in concentration of inorganic phosphate, the latter was rendered increasingly non-diffusible.

When strong calcium solutions are injected into the veins of living animals, the addition of calcium to serum takes place under circumstances similar to those in which the erratic results described above were obtained. It was therefore considered desirable to compare the serum-calcium value as determined by direct precipitation with that obtained by some reliable fundamental method, in blood abstracted from a living animal a short time after an intravenous injection of a strong calcium solution had been made. This experiment was of further interest in that Heubner [1925] found that after the injection into cats of calcium lactate, as well as of calcium hexosephosphate, only a part of the calcium could be precipitated directly as oxalate.

Accordingly (Exp. 1, Table I) a rabbit was put under open ether anaesthesia,

70 mg. of calcium as calcium levulinate in 5 cc. of solution were injected into the external jugular vein, and 5 minutes later about 30 cc. of blood were obtained rapidly from the carotid artery. In the heparin plasma obtained from the blood, the calcium was determined both by the Clark and Collip method, and also by the wet combustion method recently described by Widmark and Vahlquist [1931]. (The use of the filter-stick was replaced by the usual Clark and Collip technique, after precipitation in the cold and standing overnight.)

It will be seen (Table I) that the Clark and Collip method gave a value for the plasma-calcium 2·2 % higher than that obtained by the incineration method. We may therefore conclude that under these conditions the direct precipitation method is competent to give an accurate measurement of the plasma-calcium.

Table I. *Exp. 1.*

Weight of rabbit = 2·80 kg. Ether anaesthesia.
Injection = 70 mg. of calcium as calcium levulinate in 5 cc. solution.
Blood taken 5 mins. after injection.
Haematocrit reading: (mean of 4) 30 % corpuscles.
Plasma-calcium (Clark and Collip technique):
27·8, 27·5, 27·4; mean 27·6 mg./100 cc.
Plasma-calcium (Widmark and Vahlquist ashing technique):
27·0, 27·1, 26·9; mean 27·0 mg./100 cc.
Whole blood-calcium (Widmark and Vahlquist ashing technique):
20·0, 19·6, 19·1; mean 19·6 mg./100 cc.
70 % × 27·0 = 18·9 mg./100 cc.
Difference = 0·7 mg./100 cc.

(Blank tests: 0·0056 mg. Ca per cc. nitric acid: no allowance necessary for other reagents.)

Two further ways in which calcium in the blood may escape determination by the serum-calcium analysis may be envisaged: it may pass into the corpuscles, or it may be present as a suspension of insoluble calcium salt which would be carried down with the corpuscles. To discover whether either of these events occurred, in the above experiment the calcium content of the whole blood was determined by the Widmark and Vahlquist [1931] method and haematocrit readings were taken. It will be seen (Table I) that the calcium in whole blood is greater than the amount calculated from the ashed plasma value by 0·7 mg./100 cc. or 3·6 %. This may well be within the limits of experimental error. Heubner and Rona [1919, 1] found quantities of calcium in the corpuscles of calcium-injected animals varying from 0 to 7 mg./100 cc.; but it may be mentioned that even with normal blood there is still controversy as to the calcium content of the corpuscles, two of the latest determinations giving zero [Leiboff, 1930] and varying quantities up to 2 mg./100 cc. [Guillaumin, 1930].

From Exp. 2 (Table II) more data may be obtained. Here the whole blood-calcium was 0·7 mg./100 cc. lower than the value calculated from the plasma content. The latter value however was determined by direct precipitation which we have seen gives slightly higher results. At any rate we may conclude from

Table II. *Exp. 2.*

Weight of rabbit = 3·12 kg. Ether anaesthesia.
Injection as in Exp. 1, zero time to 0 min. 30 secs.
Bleeding, 1 min. 15 secs. to 3 mins. 15 secs.
Mean haematocrit reading = 73 %.
Blood-calcium (ashing technique):
21·9, 22·0, 22·2; mean 22·0 mg./100 cc.
Plasma-calcium (Clark and Collip):
15 mins. after drawing blood, 31·0, 31·0 mg./100 cc.
3 hrs. 30 mins. after drawing blood, 31·0, 31·4 mg./100 cc.
73 % of 31·1 mg./100 cc. = 22·7 mg./100 cc.

these experiments that the quantity of calcium which under the circumstances described is carried down with or inside the corpuscles on centrifuging is negligible.

Lastly, although we have no evidence of precipitation *in vivo* from the above experiments, it seemed desirable to discover whether calcium precipitated from the blood on standing *in vitro* at room temperature. Although Greenberg and Gunther [1930] had shown that with normal ox serum the serum-calcium value obtained is independent of the time (up to the 22 hours studied) during which the serum stands in contact with the clot, it did not follow that the serum of our experiments, so heavily charged with calcium, would be equally stable. The blood was heparinised, a portion rapidly centrifuged and the plasma-calcium determined as soon as possible. A second portion was kept, loosely corked, for 3½ hours, after which it was centrifuged at high speed for 15 minutes, and the plasma-calcium again determined. The value was found to be no lower, and to agree with the whole blood value on the assumption that there was no calcium in the corpuscles.

From the above experiments it may be concluded that the serum-calcium value as determined by the direct precipitation method gives an accurate measure of the blood-calcium after the intravenous injection of calcium levulinate.

Study of disappearance of calcium from the blood-stream.

Control experiments. In order satisfactorily to construct a curve showing the fall of serum-calcium after the injection, it was necessary to make sure that the serum-calcium returned finally to a constant level. In the curves constructed by previous authors, the serum-calcium is usually still falling when it reaches the initial level again. Accordingly attention was directed towards finding conditions under which, in the absence of injection, but with the degree of haemorrhage necessary for the withdrawal of the blood samples, the serum-calcium would stay constant for a length of time equal to the duration of the experiment.

Now the serum-calcium of the rabbit (which animal was used for these experiments as it was the most convenient) is by no means as constant as that of man or of other animals such as dogs. Thus Derevici [1929] gives the

serum-calcium of rabbits as 12–20 mg./100 cc. Most notable is the raising of the serum-calcium of the fasting rabbit by the ingestion of cabbage [Culhane, 1927, 1930]. Although this was disputed by Kapsinow and Underhill [1929], the writer has found a systematic difference between the serum-calcium levels of rabbits which have been continuously fed on cabbage and those of rabbits which have not. Thus the values for 23 cabbage-fed rabbits ranged from 14·7 to 16·9 (average 15·6), while the values for 18 rabbits of the second group, which had been fed on oats or on bran or which had taken no food since the previous evening, ranged from 8·2 to 14·9 (average 11·6) mg./100 cc. There is less variation in the cabbage-fed group. Rabbits kept continuously on this diet have a steady blood-calcium, as was shown by Davies, Dickens and Dodds [1926]. For the present purpose it was therefore decided to keep cabbage continuously in the cage both during the experiment and for at least 3 hours before. With these conditions, control experiments involving bleedings at the times to be used in the succeeding experiments showed a satisfactory steadiness of the serum-calcium, as in Exp. 3 (Table III).

Table III. *Exp. 3.*

Rabbit, female, 2·50 kg.

Time ...	0	5	20	40	60	120	180 mins.
Serum-calcium	15·0	14·6	14·5	14·2	14·1	14·4	14·5 mg./100 cc.

Technique.

The injection was made into the peripheral vein of the left ear. A puncture was made with a needle of triangular section in the peripheral vein of the right ear, and the blood allowed to flow into a centrifuge-tube where it clotted; the serum-calcium was then determined by the Clark and Collip technique. 5 cc. of blood were usually obtained in less than a minute, but in the later stages of the experiments a longer time was often consumed. The mid-time of the bleeding was noted. In these experiments no blood was taken before 5 minutes after the injection, as owing to the rapid fall of blood-calcium at this stage, the comparatively long times taken over the bleeding would introduce large inaccuracies.

Table IV. *Exp. 4.*

Rabbit, female, 3·32 kg. Dose = 13·6 mg. Ca in 1 cc. per kg., as calcium levulinate.

Time ...	(Initial)	0	5	20	43	65	125	270 mins.
Serum-calcium	14·7	(Injection)	22·9	19·6	17·0	15·9	14·9	15·1 mg./100 cc.
Excess above final level	—	—	7·9	4·6	2·0	0·9	—	— ,,
Log_e (excess)	—	—	2·07	1·53	0·69	−0·11	—	—
Antilog *A*	—	—	9·5	9·5	9·4	9·3	—	—

Table V. *Values of antilog* A.

Blood sample no. ...	1	2	3	4	5
Exp. 4	9·5	9·5	9·4	9·3	—
,, 5	11·2	10·2	13·8	10·5	—
,, 6	10·7	10·7	9·6	10·5	11·0
,, 7	10·7	10·2	10·7	10·0	—

Table VI.

Exp. no.	Weight (kg.) and sex	Dose (mg. Ca per kg.) and salt	k	Approximate time to reach constant level (mins.)	Antilog A
4	3·32 (F)	13·6 (L)	0·037	110	9·5
5	3·18 (M)	16·0 (L)	0·036	120	11·2
6	3·18 (M)	13·6 (L)	0·021	210	10·3
7	3·36 (M)	14·5 (C)	0·037	150	10·6
8	3·04 (M)	16·0 (L)	(0·086)	40	(8·5)
9	3·10 (M)	14·5 (C)	(0·047)	80	(9·1)
10 Cat*	2·28 (M)	16·0 (L)	0·023	140	7·6

L = Calcium levulinate. C = Calcium chloride. * Under chloralose anaesthesia.

Table VII.

Exp. 11. Rabbit 3·0 kg. Injection 13·1 mg. Ca per kg. as 10 % levulinate.
Inorganic phosphate* in (citrated) plasma:
Before injection 4·0 mg. P/100 cc.
5–6 mins. after injection 4·7 „

Exp. 12. Rabbit 3·04 kg. Injection as in Exp. 11.
Inorganic phosphate in serum:
Before injection 4·5 mg. P/100 cc.
4–5 mins. after injection 4·9 „

* de Wesselow [1924].

Table VIII.

Exp. 13. Rabbit, 3·03 kg. Injection 31 mg. Ca per kg. as calcium chloride.
Haematocrit reading: 75 % plasma.

	Serum-calcium*	Haemoglobin†	Alkali reserve‡
Before injection	14·2	100 units	29
3–6 mins. after injection	32·0	92·6 units	25
Increase	17·8	−7·4 %	−4
	mg./100 cc.		cc. CO_2/100 cc.

4 cc. CO_2 at N.T.P. ≡ 3·6 mg. Ca

* Clark and Collip [1925]. † Myers [1924]. ‡ Van Slyke [1917].

Results.

The results are included in Tables IV–VI. A typical curve is shown in Fig. 1, in which the serum-calcium values found in Exp. 4 are plotted against

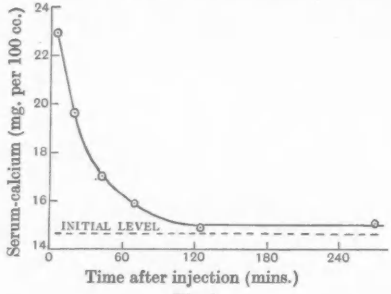


Fig. 1.

the time after the injection. The curve is concave upwards, thus differing from the curves given by Heténi [1924], but agreeing with those of Gersch-

mann [1930] and Rothlin [1930]. It will be observed that the final steady level differs little from the initial level: this was not so in all the experiments, the final level being sometimes higher and sometimes lower than the initial level. The greatest observed difference between the two was, however, only 1·4 mg./100 cc. There was one experiment (not included in the Tables) in which the serum-calcium did not return to a constant level, but fell steadily after reaching the initial value. In all the experiments doses of the same order of magnitude were given.

Discussion.

It is of interest to see whether there is any approximate relation between the excess of the serum-calcium at any instant above the final constant level and the time to which it corresponds. In the third row of Table IV the values

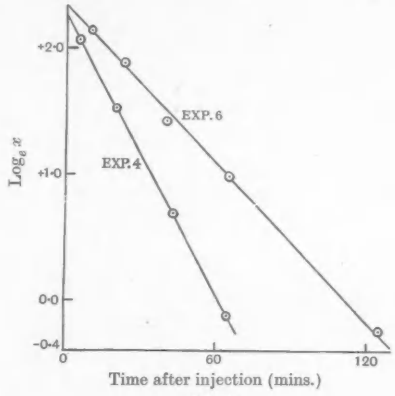


Fig. 2.

of this excess (x) are given for each point of Exp. 4. In Fig. 2 $\log_e x$ is plotted against the time after the injection (t), and it will be seen that the points lie on a straight line. x and t are therefore connected by the relation

$$\log_e x = A - kt,$$

where k and A are constants for the particular experiment. Hence the rate of fall of calcium excess at any instant is directly proportional to the excess obtaining at that instant. A is the logarithm of the (theoretical) initial value of the excess, while k is the rate of fall per unit calcium excess (for Exp. 4, 0·037 mg./100 cc. per minute per mg./100 cc. excess).

In order to determine how closely the data fit this logarithmic law, we may insert in the above equation the values for x, t and k, and so calculate A for each point of the curve. From the constancy of A we may draw conclusions concerning the accuracy with which the law is obeyed. The corresponding values of antilog A for the points of Exp. 4 are given in the fifth

row of Table IV, and the constants for significant analyses in other rabbit experiments are given in Table V.

Out of the experiments performed, three satisfactory curves were obtained with calcium levulinate and one with calcium chloride. In addition there were some experiments in which the rate of fall of serum-calcium was so rapid that enough points on the significant part of the curve were not obtained to give logarithmic graphs of interest (data given in brackets in Table VI, in which the results of the important experiments are summarised).

It must be remembered, in interpreting the results obtained, that plotting data on a logarithmic scale tends to smooth out divergences; but an examination of Fig. 2 and Table V shows that the data are distributed evenly about the curve representing the logarithmic law, and do not show any systematically increasing divergence. It would of course be idle to suggest that the relationship is exactly accurate or to attach any physical significance to it, for we are dealing with the net result of several complicated processes and the return to a dynamic equilibrium. We can, however, say that the data are conveniently represented by this equation, and that the rate of disappearance of serum-calcium at any instant is approximately proportional to the excess above the steady value which obtains at that instant. Such a formulation has indeed previously been employed in the kinetics of blood chemistry: for example Fishberg [1930] found that the disappearance of foreign sugars from the blood-stream can be described by this (to use a chemical analogy) "unimolecular law."

It will be seen from Table VI, from the values of k and of the approximate time taken for the serum-calcium to return to a constant level, that the rate at which calcium disappears from the blood-stream varies greatly among the different animals. Exp. 7 with chloride yields results which fall in line with those with levulinate [*cf.* Rothlin, 1930]; there might well have been a difference since calcium chloride is said to disturb the acid-base equilibrium of the blood.

Interest attaches to the constant "antilog A." This initial highest value of the serum-calcium is never of course realised in practice owing to disappearance of calcium from the blood-stream during the injection, and also because the injected calcium takes some time to become completely mixed with the circulating blood [*v.* Erlanger, 1921]. But it gives an approximation to the amount of serum-calcium which would disappear during the course of the experiment if the fall of serum-calcium during the first 5 minutes were to exhibit the same relationships as it does during the later part of the experiment. It is found that the loss calculated for the whole experiment is quite inadequate to account for the total disappearance. Thus in Exp. 4, 13·6 mg. of calcium per kg. are injected. Now from the table given by Erlanger [1921] it appears that 50 cc. is a reasonable figure to take for the blood-volume per kg. of rabbit, and as 70 % is a usual haematocrit reading for rabbit's blood we may suppose that there are 35 cc. of serum per kg. The potential increase

in serum-calcium is therefore $\frac{13{\cdot}6 \times 100}{35} = 39$ mg./100 cc. But antilog A is only 9·5. Hence about 30 mg./100 cc. have not been accounted for. Even if we take the improbable figure of 9 % for the blood-volume, the unexplained disappearance amounts to 12 mg./100 cc., a quantity greater than the total loss calculated by extrapolation from the curve. Obviously, in addition to the disappearance of serum-calcium at a rate expressed approximately by the logarithmic relationship, there is a rapid disappearance immediately after the injection. This idea is borne out by a curve given by Rothlin [1930]; following injection of 29 mg. Ca per kg. the rate of fall during the first 20 minutes is relatively very much greater than that during the succeeding 100 minutes. It is reasonable to suppose that two different processes take place.

The analyses of Heubner and Rona [1919, 2; 1923], Jungmann and Samter [1924], Hecht [1924] and Heubner [1925] showed that, after toxic doses, the only significant rise in the calcium content of the tissues occurred in the kidneys. This, together with the calcium in urine and intestinal contents, did not account, a few hours after the injection, for the large disappearance of injected calcium from the blood-stream: deposition of calcium in the bones seemed the only explanation. Hetényi [1924], in an animal experiment, recovered after 3 hours only 15·6 % of the injected calcium from urine and faeces. In the same animal, before and after injecting a lethal dose (294 mg. per kg.) of calcium, he obtained a 2 % increase in the calcium concentration of the bone, which may be significant.

Dadlez [1926] found that the urinary calcium excretion was abnormally high at the end of 6 hours after injection. Brull [1930] on the other hand [*cf.* also Proverman and Brull, 1930] considers that the renal calcium excretion following intravenous calcium chloride injection is dependent entirely on the rate of flow of urine, thereby differing from Hetényi and Nógrádi [1925], who found in normal human beings a trebled calcium concentration in the urine during the first 30 minutes after injection. Recently Taylor and Fine [1930], by washing out loops of isolated intestine [*cf.* Stewart and Percival, 1927], found that the intestinal calcium excretion does not rise above the small normal value during the first 3 hours. They confirmed the idea that the calcium does not leave the body at first by observing that in eviscerated and nephrectomised animals the fall of blood-calcium was just "as abrupt and profound" as in normal animals.

It thus appears probable that during the first 3 hours most of the injected calcium is deposited on the bones, but we have yet to explain the large disappearance in the first few minutes.

Precipitation as phosphate following toxic doses is envisaged by Heubner [1923, 1931]. On the other hand, while injection of phosphate causes a hypocalcaemia [Binger, 1917], large injections of calcium actually cause a rise in inorganic phosphate [Salvesen, Hastings and MacIntosh, 1924; Collip, 1926]. Brull [1930] added calcium chloride to serum circulating in a Starling

heart-lung preparation: there was no precipitation or alteration of the inorganic phosphate; some of the added calcium became non-diffusible. The writer has shown *in vivo* (Table VII) that a few minutes after the injection of calcium levulinate the inorganic phosphate is not lowered. Earlier in the paper it was shown that there was no calcium in the form of a suspension, which can be centrifuged. There seems, therefore, no evidence of the immediate precipitation of calcium phosphate.

The immediate precipitation of injected calcium chloride as carbonate according to the equation

$$CaCl_2 + 2NaHCO_3 \rightarrow 2NaCl + CaCO_3 + CO_2 + H_2O$$

would of course cause a decrease of alkali reserve. Acidosis is said to be caused by injection of calcium chloride owing to excretion of calcium through the intestine [Mainzer, 1927]. In Exp. 13 (Table VIII) about 5 minutes after a large injection of calcium chloride the alkali reserve had diminished by 14 %, corresponding to a precipitation of 3·6 mg. Ca/100 cc. serum. This figure is however too high, as a dilution of the blood of at least 7·4 % had occurred (haemoglobin comparison). Using the high figure of 9 % for the blood-volume, 28 mg. Ca/100 cc. had disappeared in the 5 minutes. There is clearly little precipitation as carbonate.

There remains the possibility of immediate rapid diffusion into the tissue spaces. Following the work of Chiari and Januschke [1910] and Meyer [1910], it was believed that calcium decreased the permeability of the capillary walls; that such is not the case to any extent has been shown by Yanagawa [1916], Bayliss [1918], and Smith and Mendel [1920]. That calcium can readily penetrate the capillary walls is shown by the appearance of calcium in oedema fluid and serous transudates; while Arnold and Mendel [1927] concluded that calcium passes easily and rapidly at all times between blood, lymph and tissues.

We are thus led to picture the following events: immediately following the injection of hypertonic calcium salt solutions into the blood-stream, calcium diffuses into the tissue spaces until there is some form of equilibrium between the calcium in these spaces and the raised blood-calcium. The added calcium now disappears from the tissues and from the blood-stream, the rate of disappearance from the latter being approximately proportional at any instant to the excess of blood-calcium above the normal value, until at the end of 2–3 hours (when the tissue analyses of Heubner and Rona, *etc.* were made) the calcium content of tissues and blood has returned to normal. The calcium appears for the most part to be deposited in the bones, but a smaller quantity is probably simultaneously excreted.

There is some doubt concerning the fate of the injected calcium after this, for varying results have been obtained. Dadlez [1926] claimed that the retention of calcium in tissues or bones immediately following the injection is only temporary, a complete excretion of the injected calcium being achieved

in 24 hours. On the other hand, Greenwald and Gross [1925] recovered in 6 days only 0·40 g. of the 0·76 g. which was injected, while Salvesen, Hastings and MacIntosh [1924] even state that the faecal excretion of calcium during the 6 days following a large injection of calcium chloride is lower than that before the injection. Presumably, the relation of the amount of calcium in the diet to the demands of the animal is a factor which has a profound influence in this matter.

Summary.

1. The serum-calcium as determined by direct precipitation can give an accurate measure of the calcium content of the blood following the intravenous injection of calcium levulinate.

2. Following the intravenous injection of calcium chloride and of calcium levulinate into cabbage-fed rabbits, it was found that the rate of fall of serum-calcium at any time greater than 5 minutes after the injection was approximately proportional to the excess at that time of the serum-calcium above the final constant level. In addition, there was a large disappearance of serum-calcium during the first 5 minutes not accounted for by this relationship.

3. The bearing of the experimental results upon our knowledge of the fate of intravenously injected calcium is discussed, and a description is given of the events which probably occur.

It is a great pleasure to acknowledge the interest taken by Prof. E. C. Dodds in these experiments.

The work was done under a research grant from the Crookes Laboratories, for which the writer wishes to make acknowledgment here.

REFERENCES.

Arnold and Mendel (1927). *J. Biol. Chem.* **72**, 189.
Bayliss (1918). Intravenous injection in wound shock, p. 34. (Longmans, Green and Co., London.)
Bennett (1931). *Lancet*, ii, 403.
Binger (1917). *J. Pharm. Exp. Ther.* **10**, 105.
Brull (1930). *Arch. Internat. Physiol.* **32**, 138.
Chiari and Januschke (1910). *Wien. klin. Woch.* **23**, 427.
Clark (1920). *J. Biol. Chem.* **43**, 89.
—— and Collip (1925). *J. Biol. Chem.* **63**, 462.
Collip (1926). *Amer. J. Physiol.* **76**, 472.
Condorelli (1926). *Arch. Farm. Sper.* **41**, 122.
—— (1927). *Arch. Farm. Sper.* **43**, 88.
Conrad (1878). *Ber. deutsch. chem. Ges.* **11**, 2177.
Culhane (1927). *Biochem. J.* **21**, 1015.
—— (1930). *J. Biol. Chem.* **86**, 113.
Dadlez (1926). *Biochem. Z.* **171**, 146.
Davies, Dickens and Dodds (1926). *Biochem. J.* **20**, 695.
Derevici (1929). *Compt. Rend. Soc. Biol.* **100**, 925.
Dodds and Greville (1931). *Brit. Med. J.* ii, 190.
Erlanger (1921). *Physiol. Rev.* **1**, 177.
Fishberg (1930). *J. Biol. Chem.* **86**, 665.

Gerschmann (1930). *Compt. Rend. Soc. Biol.* **104**, 413.
Greenberg and Gunther (1930). *J. Biol. Chem.* **85**, 491.
Greenwald and Gross (1925). *J. Biol. Chem.* **66**, 201.
Grollman (1927). *J. Biol. Chem.* **72**, 565.
Guillaumin (1930). *Bull. Soc. Chim. Biol.* **12**, 1269.
Hecht (1924). *Biochem. Z.* **144**, 270.
Hendriks (1929). *Biochem. J.* **23**, 1206.
Hetényi (1924). *Z. ges. exp. Med.* **43**, 123.
—— and Nógrádi (1925). *Klin. Woch.* **4**, 1308.
Heubner (1923). *Klin. Woch.* **2**, 1603.
—— (1925). *Biochem. Z.* **156**, 171.
—— (1931). *Biochem. Z.* **234**, 221.
—— and Rona (1919, 1). *Biochem. Z.* **93**, 187.
—— —— (1919, 2). *Biochem. Z.* **93**, 353.
—— —— (1923). *Biochem. Z.* **135**, 248.
Jungmann and Samter (1924). *Biochem. Z.* **144**, 265.
Kapsinow and Underhill (1929). *J. Biol. Chem.* **82**, 377.
Kramer and Tisdall (1921). *J. Biol. Chem.* **47**, 475.
Leiboff (1930). *J. Biol. Chem.* **85**, 759.
Loucks and Scott (1929–30). *Amer. J. Physiol.* **91**, 27.
Mainzer (1927). *Klin. Woch.* **6**, 1689.
Meyer (1910). *Münch. med. Woch.* 2277.
Myers (1924). Practical chemical analysis of blood. (Kimpton, London.)
Proverman and Brull (1930). *Bull. Soc. Chim. Biol.* **12**, 1151.
Rothlin (1930). *Z. ges. exp. Med.* **70**, 634.
Salvesen, Hastings and MacIntosh (1924). *J. Biol. Chem.* **60**, 327.
Smith and Mendel (1920). *Amer. J. Physiol.* **53**, 323.
Stewart and Percival (1927). *Biochem. J.* **21**, 301.
Taylor and Fine (1930). *Amer. J. Physiol.* **93**, 544.
Van Slyke (1917). *J. Biol. Chem.* **32**, 455.
—— and Sendroy (1929). *J. Biol. Chem.* **84**, 217.
de Wesselow (1924). Chemistry of the blood in clinical medicine, p. 244. (Benn, London.)
Widmark and Vahlquist (1931). *Biochem. Z.* **230**, 245.
Yanagawa (1916). *J. Pharm. Exp. Ther.* **9**, 75.

CCVIII. THE ABSORPTION OF WATER BY GELATIN.

PART VII. THE INFLUENCE OF TEMPERATURE ON SWELLING IN ACID SOLUTIONS AND THE COMBINATION OF GELATIN WITH HYDROCHLORIC, NITRIC OR SULPHURIC ACID.

By WINNIFRED BERTHA PLEASS.

From the Laboratories of the British Leather Manufacturers' Research Association.

(*Received October 12th, 1931.*)

It has long been known that the influence of aqueous solutions of inorganic substances on the physical properties of proteins is modified by the nature of the ions present. Loeb [1922] has stated that at the same p_H dibasic acids and diacidic bases induce only half as much water absorption by gelatin as monoacidic bases and monobasic acids.

The simple mathematical relationship postulated by Loeb has been denied by various workers: Ostwald, Kuhn and Böhme [1925], investigating the swelling of gelatin, found that the volume at maximum swelling was not related to the valency of the anion of the acid; Küntzel [1926], working on the contraction of tendons from the tail of the rat in acid solutions, and also the author [1929], working on the increase in weight of purified leaf gelatin, found that at the same p_H the influence of nitric acid is slightly greater than that of hydrochloric acid, which in turn is considerably greater than that of sulphuric acid at $p_H < 3{\cdot}2$. In very dilute solutions however no difference in the effects of the three acids can be distinguished. Isgarischev and Pomeranzeva [1926] have measured the swelling of powdered caseinogen in $N/20$ solutions of a large number of organic acids and suggest that the predominating factor is the nature and structure of the anion of the acid.

The author [Pleass, 1929] discussed the question of the magnitude of the swelling and also reported that the p_H for maximum swelling was determined by the anion of the acid. Recently published work [Jordan-Lloyd, 1930; Pleass, 1930] has shown that, although this p_H in solutions of hydrochloric acid is independent of the temperature between 0° and 18°, in solutions of sodium hydroxide and calcium hydroxide the p_H for maximum swelling becomes greater as the temperature is decreased. It was decided, therefore, that the influence of temperature on solutions of sulphuric acid should also be investigated.

The experimental material and method were exactly similar to that previously described [Jordan-Lloyd and Pleass, 1927; Pleass, 1930]. Pieces of purified leaf gelatin were weighed and immersed in solutions of sulphuric acid of varying concentrations at constant temperatures.

After 7 days' immersion at 0°, 4 days at 12°, or 3 days at 18° or 25°, the pieces of gelatin were removed from the solutions, lightly blotted and weighed. The final weights are expressed as a percentage of the initial weight, corrected to its weight after drying at 100°. The figures for the percentage weights of the gelatin are plotted graphically against the final p_H of the solutions.

In Fig. 1 are plotted the curves for the water absorption of gelatin from solutions of sulphuric acid of final p_H 0·1–5·0 at temperatures of 0°, 12°, 18° and 25°.

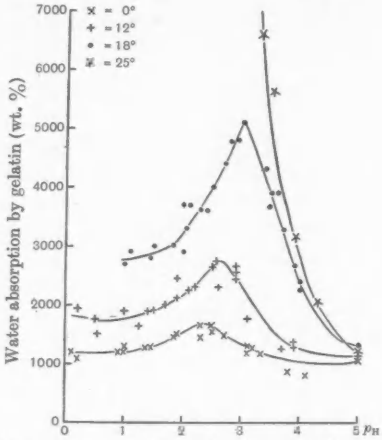


Fig. 1. The influence of temperature and p_H on the absorption of water by gelatin in solutions of sulphuric acid.

The first inspection of the curves shows that the lower the temperature, the less the water absorption. At 25°, as the p_H moves from 5·0 to 3·3, the water absorption of the gelatin increases to so great a degree that in the more concentrated solutions of sulphuric acid the gelatin is too soft to weigh. At 12° and 0°, the degree of swelling is much less than at 18°, and also at the lower temperatures the peak of maximum swelling is less sharply defined. Over the whole range of p_H studied, the magnitude of the swelling lies between 1000 and 1800 at 0°, 1100 and 2800 at 12° and 1300 and 5100 at 18°. At 0° and 12°, the gelatin does not dissolve in solutions of sulphuric acid as acid as p_H 0·1. In an analogous manner, Jordan-Lloyd [1930] found that gelatin did not dissolve in *N* hydrochloric acid at 0°, but at 18° the gelatin dissolved in all solutions of $p_H < 0·9$.

A closer examination of the curves reveals that not only is the degree of swelling and the sharpness of the peak dependent on the temperature, but

that the maximum occurs at p_H 3·0 at 18°, at p_H 2·6 at 12° and at p_H 2·4 at 0°.

The fact that the p_H for maximum swelling of gelatin in solutions of sulphuric acid varies with temperature in an analogous manner to the alteration in solutions of sodium and calcium hydroxides, while the p_H for maximum swelling in solutions of hydrochloric acid is independent of temperature (from 0 to 18°) is very striking. Jordan-Lloyd [1930] and the author [Pleass, 1930] suggested that in the alkaline systems this phenomenon might be due to some structural change in the gelatin molecule, possibly hydrolysis, which proceeded to a greater extent at the higher temperature. This opinion was based largely on the fact that gels containing sodium hydroxide decrease in p_H on keeping for several days at 18°, even when atmospheric carbon dioxide is rigidly excluded. In order to investigate more fully the possibility of the

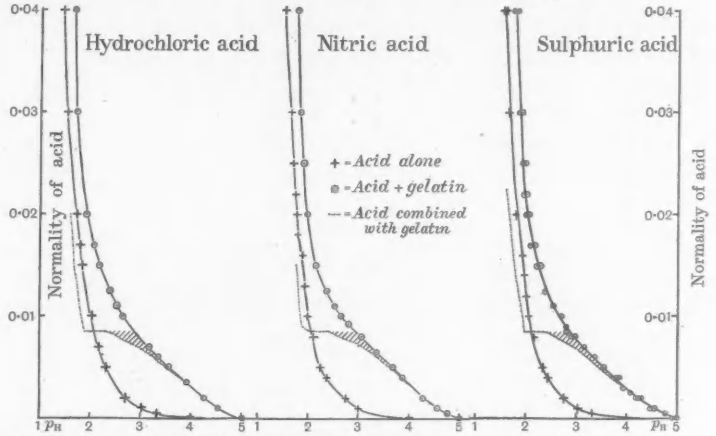


Fig. 2. Titration and acid fixation curves of a 1 % solution of gelatin with hydrochloric, nitric or sulphuric acid at 18–20°.

hydrolysis of gelatin occurring in the presence of mineral acids, the p_H of a 1 % solution of gelatin containing various known quantities of hydrochloric, nitric or sulphuric acid were determined by means of the glass electrode and plotted against the normality of the acids present (see Fig. 2) and after keeping for a week at 18–20° these gels were re-examined. It was found that within the limits of the experimental error no changes in the p_H were detected. Thus, no evidence was obtained of hydrolysis of the gelatin on keeping in any of the acid solutions.

The p_H of solutions of the acids alone were also measured and the results are plotted against the corresponding normality of the acids (Fig. 2). The amount of acid fixed by the gelatin has been derived by plotting the differences between the curve for the acids alone and the curve for acid in the presence of the gelatin at each p_H. The curves for the combination of acid are

remarkably similar for the three acids examined—in fact they are probably identical within the experimental error of the method. With all three acids, a flat portion of the curve occurs from about p_H 2·4 to 1·9 and at a normality of acid of 0·0085. This indicates that the combining weight of gelatin is the same for the three acids (approximately 1180) from p_H 2·4 to 1·9. Assuming that the gelatin behaves as a weak base and combines with strong acids in accordance with the following equation in solutions of $p_H > 1{\cdot}9$

$$\text{Gelatin} + \text{HX} \rightleftharpoons \text{Gelatin HX},$$

therefore

$$\frac{[\text{G}][\text{HX}]}{[\text{G.HX}]} = \text{K}.$$

In very dilute solutions, the reaction will proceed predominantly from left to right since there is an excess of gelatin. That is, most of the acid is in combination with the gelatin (*cf.* Fig. 2 where the curve for gelatin and acid is almost superimposed on the curve for gelatin combined with acid).

In moderately dilute acid solutions, say p_H 3·5–2·5, the reaction proceeds in both directions since there is neither an excess of acid nor of gelatin to force the reaction to proceed to completion. An indication of the degree of dissociation of the gelatin-acid compound is given by the shaded area in the graphs (Fig. 2).

In more concentrated acid solutions (p_H 2·5–1·9), the excess of acid causes the reaction to proceed from left to right. The flatness of the dotted curve over this range shows that the full quota of acid for the complete neutralisation of the most strongly basic groups (probably the amino-groups) has been taken up. It can be seen that half neutralisation of the gelatin occurs at about p_H 3·7 from which it may be deduced that the first basic dissociation constant is 5×10^{-11}. This value is identical within the limits of experimental error with that obtained by Atkin and Douglas [1924] working at 24° and using the hydrogen electrode for the determinations of the p_H. In strongly acid solutions (p_H 1·9) the gelatin takes up more acid, probably by means of the acid combining with the weaker basic groups (possibly the imino-groups). This is in agreement with the work of Jordan-Lloyd and Mayes [1922] and Atkin and Douglas [1924].

Thus the p_H for maximum swelling of gelatin in solutions of hydrochloric and nitric acids at temperatures between 0° and 18° and in solutions of sulphuric acid at 0° approximates very nearly to the highest p_H at which the first stage of fixation of these acids by the gelatin at 18–20° is complete, namely p_H 2·4. Since the combination of gelatin with all three acids occurs stoichiometrically at 18–20°, the differences in the magnitude of the swelling and the p_H for maximum swelling of the gelatin in solutions of the three acids at this temperature may possibly be explained by the specific characteristics of the anions and their influence on the physico-chemical properties of water, since these differences become less as the temperature is decreased until at 0°, the freezing point of water, they are negligible.

Jordan-Lloyd has recently [1931] established that the magnitude of the water absorption of both dried and undried gelatin gels is proportional to the square of the centigrade temperature from 0 to 18° for all the external conditions studied, *i.e.* water, solutions of acids, bases and salts. She develops the idea that the influence of temperature on the activity of the water, one of the common factors in all the systems, may be the direct cause of the influence of temperature on swelling. She emphasises the point that the swelling of the gelatin is proportional to the square of the degrees on a scale of which the zero is based on a property of water.

As previously indicated [Pleass, 1930], at 0° at the p_H of maximum swelling in hydrochloric and sulphuric acids, the hydrogen ion concentration is almost equal to that of the hydroxyl ion concentration at which maximum swelling occurs at 0° in the alkaline system (p_H 11·5–11·6). On superficial examination, therefore, it would appear that, assuming that the p_H of the solutions in which maximum swelling of the gelatin occurs gives an indication of the basicity of the gelatin in acid solutions and of the acidity of the gelatin in alkaline solutions, the isoelectric point of the gelatin should coincide with the p_H of neutrality. Owing, however, to the logarithmic nature of the p_H scale, small variations in the ionic concentration at the positions of maximum swelling are inappreciable, while similar variations in ionic concentration are of considerable magnitude near the neutral point when expressed on the p_H scale.

It should also be indicated that in all the experiments on the swelling of gelatin dealt with in this series of papers, the p_H values considered have been those of the external solutions, since the determination of the p_H of the internal gel involves the melting of the gel and may be attended by unknown errors. It is probable however that an accurate knowledge of the internal p_H of the gel would be very valuable when correlating the swelling of gelatin with the combination of gelatin with acids and alkalis.

Summary.

The water absorption of purified leaf gelatin in solutions of sulphuric acid from p_H 0·1 to 5 at 0°, 12°, 18° and 25° has been studied. Increase in temperature causes an increase in water absorption until at 25° in all solutions more acid than p_H 3·2 the gelatin is too soft to weigh.

Maximum swelling occurs at p_H 3·0 at 18°, p_H 2·6 at 12° and p_H 2·4 at 0°.

Curves for the combination of gelatin with hydrochloric, nitric and sulphuric acids from p_H 5·0 to 1·7 are given. The three curves are practically identical, exhibiting a flat portion from p_H 2·4 to 1·9, and indicate a combining weight for gelatin of 1180 and a first basic dissociation constant of 5×10^{-11}.

No evidence of hydrolysis of gelatin in these acid solutions (*i.e.* $p_H > 1·5$ at 18–20°) has been obtained.

The author wishes to express her thanks to the Council of the British Leather Manufacturers' Research Association for permission to publish this work and to the Director, Dr Jordan-Lloyd, for her continued interest and advice.

REFERENCES.

Atkin and Douglas (1924). *J. Soc. Leather Trades' Chem.* **8**, 359.
Isgarischev and Pomeranzeva (1926). *Koll. Z.* **38**, 235.
Jordan-Lloyd (1930). *Biochem. J.* **24**, 1460.
—— (1931). *Biochem. J.* **25**, 1580.
—— and Mayes (1922). *Proc. Roy. Soc. Lond.* B **93**, 69.
—— and Pleass (1927). *Biochem. J.* **21**, 1352.
Küntzel (1926). *Koll. Z.* **40**, 264.
Loeb (1922). Proteins and the theory of colloidal behaviour. (New York.)
Ostwald, Kuhn and Böhme (1925). *Koll. Chem. Beihefte*, **20**, 412.
Pleass (1929). *Biochem. J.* **23**, 358.
—— (1930). *Biochem. J.* **24**, 1472.

CCIX. A METHOD FOR DETERMINING ALUMINIUM IN CERTAIN ALUMINIUM PROTEIN COMPOUNDS.

By CLIFFORD GEORGE POPE.

From the Wellcome Physiological Research Laboratories, Beckenham, Kent.

(*Received October 13th, 1931.*)

In connection with the work which has been carried out at these Laboratories on the precipitation of diphtheria toxoid by various aluminium salts [Glenny, Pope, Waddington and Wallace, 1926; Wallace, 1927; Glenny and Barr, 1931] it became necessary to make a number of determinations of the aluminium content of the precipitates so formed.

From an investigation of various methods which have been proposed for the estimation of aluminium we came to the conclusion that precipitation of the aluminium by use of 8-hydroxyquinoline would prove most satisfactory for our purpose. This reagent was used by Berg [1926] for the estimation of aluminium, and later Pichler [1929] reported a micro-method based on its use.

The suspensions of alum-toxoid with which we were working contained aluminium in an amount varying from a fraction of a milligram to about 1 mg. per cc. and the method was adopted for convenience for amounts varying from 0·3 to 1·0 mg. Using less than 0·3 mg. the results were not reliable, and for choice the material tested should contain between 0·5 and 1·0 mg. aluminium.

A difficulty, regarding which we can find no mention in the literature, was encountered in dealing with the aluminium complex salt, owing to the fact that the precipitate is not easily "wetted" and behaves rather like finely divided sulphur. This property made it difficult to transfer the precipitate quantitatively to a filter. While this difficulty may have been due to the sample of 8-hydroxyquinoline used, it was found that repeated crystallisation of this reagent did not obviate the trouble. At the suggestion of Dr Trevan, we tried the effect of adding a little sodium taurocholate, which proved effective.

We found it more convenient to determine the amount of "complex" formed volumetrically instead of gravimetrically. Berg titrated the complex directly with standard bromine solution using an indicator, but in our experiments better results were obtained by dissolving the complex in hydrochloric acid, adding excess of potassium bromate, and then determining the excess bromine by adding potassium iodide and titrating with thiosulphate.

At first we used 2*N* ammonium acetate following the method of Pichler [1929] in order to precipitate the complex, but we found that, unless the precipitate was washed very thoroughly, trouble was experienced owing to traces of acetate remaining in the filter and reacting with bromine during the estimation. Borax solution was found to be much more satisfactory than acetate for our purpose, and it had the added advantage that it dissolved the alum-toxoid precipitates and so obviated the necessity for incineration with sulphuric acid. Further, as the solubility of 8-hydroxyquinoline was greater in borax solution than in water, the precipitate when collected on the Gooch crucible was washed with borax solution to remove the excess of this reagent.

Reagents.

Standard solution of aluminium. Potassium alum (A.R.) dried in a vacuum over calcium chloride. Dissolve 1·7509 g. in distilled water and make to 100 cc. (*Note.* In working with a solution made to contain 0·1 mg. aluminium per cc. it was found that after a day or two in a volumetric flask the solution gave no reaction for aluminium when tested with aurintricarboxylic acid, using the method of Myers, Mull and Morrison [1928]. After adding one drop of concentrated H_2SO_4 the aluminium, which may have been adsorbed on the glass of the flask, was liberated and correct values for the aluminium present were obtained. In view of this experience we have made it a practice to add one drop of H_2SO_4 when making up standards.) The standard solution contains 1 mg. of aluminium per cc.

Borax solution. A 5 % solution of sodium borate (A.R.).

8-Hydroxyquinoline. A 2 % solution in absolute alcohol. 1 cc. of this reagent is sufficient to precipitate 1·2 mg. of aluminium. When 1·0 cc. of this solution is mixed with 10 cc. of 5 % borax solution, a little 8-hydroxyquinoline may separate if the temperature is below 15°. This will not affect the results if the amount of aluminium is between 0·5 and 1·0 mg. but for amounts below 0·5 mg. it is better to use 0·5 cc. of reagent.

Hydrochloric acid. Hydrochloric acid (A.R.) diluted 1 in 3. For each test 40 cc. are used.

Bromide-bromate solution. Dissolve 1·6702 g. potassium bromate (A.R.) and 10 g. of potassium bromide in water and make to 1000 cc. This solution is *M*/100 in respect to the bromate, and 10 cc. provide sufficient bromine for 1·35 mg. of aluminium as $Al(C_9H_6ON)_3$.

N/20 thiosulphate solution. Prepared and standardised in the usual manner. Each cc. of *N*/20 thiosulphate = 0·1129 mg. aluminium.

Blank titration for total bromine available.

Measure out 40 cc. of HCl reagent into a beaker and add water to about 200 cc. Add 10 cc. of bromate-bromide reagent, and after two minutes 1 g. (approx.) of potassium iodide. Titrate the liberated iodine with *N*/20 thiosulphate until almost colourless, completing after the addition of starch

indicator in the usual manner. If the bromate and thiosulphate are $M/100$ and $N/20$ respectively, 10 cc. of bromate-bromide reagent will require 12 cc. of thiosulphate.

Estimation of aluminium.

Measure out an amount of the material containing between 0·3 and 1·0 mg. (The amount present may be roughly estimated from the bulk of precipitate given with the 8-hydroxyquinoline.) Add 10 cc. of borax solution, heat to boiling-point, then add 1·0 cc. of 8-hydroxyquinoline reagent. At this stage two drops of 10 % sodium taurocholate are added to cause "wetting" of the precipitate. The tubes are now heated in a boiling water-bath to drive off the alcohol in which the precipitate is somewhat soluble. They are then cooled to room temperature.

The yellow precipitate is separated by filtration through a Gooch crucible, traces of precipitate in the tube are removed, and finally the precipitate on the crucible is washed with 5 % borax solution. The precipitate is then ready to be dissolved in HCl.

Heat 40 cc. of HCl reagent to boiling. Hold the crucible over a 400 cc. beaker and half fill it with boiling HCl. Stir gently with a small glass rod, but avoid disturbing the asbestos mat at this stage. When most of the first addition of HCl has passed through the filter, add more boiling HCl and stir gently to break up the asbestos mat. Wash through with the remainder of the 40 cc. of HCl. Finally rinse the crucible with a little distilled water, wash down the sides of the beaker and make to a total volume of about 200 cc.

Add 10 cc. of bromate-bromide solution, stir well and after two minutes add about 1·0 g. potassium iodide. Titrate with $N/20$ thiosulphate. Some caution is necessary in the titration owing to the yellow colour of the bromine derivative of 8-hydroxyquinoline, and the starch indicator should, therefore, be added earlier than usual. The amount of aluminium is obtained as follows:

$$(T_1-T_2) \times 0{\cdot}1129 \times N \text{ mg. aluminium,}$$

where

T_1 = cc. thiosulphate required for bromine blank,
T_2 = cc. thiosulphate required for excess bromine (titrated as iodine),
N = Normality factor for thiosulphate and each 1·0 cc. $N/20$ thiosulphate = 0·1129 mg. aluminium.

Experimental results.

The accuracy of the method using pure solution of potassium alum.

The stock solution used for the following tests was made up to contain 1·0 mg. aluminium per cc. From this there were prepared dilutions so that each fraction of a mg. used was contained in a volume of 1·0 cc. The results given below are for estimations carried out at different times, when pure solutions were included as controls in routine estimation.

Mg. Al used	Mg. Al found	Difference in mg.
0·2	0·203	+0·003
0·2	0·200	±0·000
0·2	0·216	+0·016
0·2	0·191	−0·009
0·2	0·195	−0·005
0·3	0·305	+0·005
0·3	0·302	+0·002
0·3	0·310	+0·010
0·3	0·306	+0·006
0·3	0·310	+0·010
0·4	0·409	+0·009
0·4	0·408	+0·008
0·4	0·410	+0·010
0·4	0·412	+0·012
0·4	0·404	+0·004
0·5	0·500	±0·000
0·5	0·500	±0·000
0·5	0·501	+0·001
0·5	0·506	+0·006
0·5	0·495	−0·004
0·5	0·493	−0·007
0·5	0·491	−0·009
1·0	0·995	−0·005
1·0	0·997	−0·003
1·0	0·997	−0·003
1·0	1·002	+0·002
1·0	0·998	−0·002
1·0	0·997	−0·003

Recovery of aluminium when mixed with C. diphtheriae *culture filtrates.*

In order to determine the accuracy of the method when organic materials were present in addition to the aluminium, the following experiment was carried out.

Known amounts of aluminium were measured into tubes, and 0·5 cc. of a *C. diphtheriae* toxin filtrate added. A precipitate formed in each. 10 cc. of borax solution were then added to each tube and the aluminium estimated in the manner described. The following results were obtained, showing that the presence of organic materials did not affect the results.

Total Al present (mg.)	Al found (mg.)	Difference (mg.)
0·3	0·303	+0·003
0·3	0·298	−0·002
0·4	0·403	+0·003
0·4	0·398	−0·002
0·5	0·501	+0·001
0·5	0·507	+0·007

In a further experiment, a typical *C. diphtheriae* culture filtrate was diluted 1 in 5 with saline and mixtures made containing varying amounts of potassium alum. In each case there was formed a precipitate, small in the low alum concentration and increasing as the alum concentration increased. From these mixtures varying volumes were measured out, precautions being taken to keep the suspensions as uniform as possible during measurement. The volumes required to give a suitable amount of aluminium for estimation varied from 25 to 2 cc. of suspension. The results show the method to be suitable for the special purpose for which it was worked out.

Potassium alum in mixture %	Vol. used for estimation cc.	Mg. Al found	Mg. Al per cc. found	Mg. Al per cc. calculated
0·05	25	0·713	0·0285	0·0285
0·1	20	1·085	0·0542	0·0590
0·2	10	1·141	0·1141	0·1140
0·3	5	0·871	0·1742	0·1710
0·4	5	1·120	0·2240	0·2280
0·5	4	1·150	0·2870	0·2850
0·5	4	1·130	0·2820	
0·6	3	1·020	0·3400	0·3420
0·7	3	1·185	0·3950	0·3990
0·8	2	0·906	0·4530	0·4560

SUMMARY.

A method suitable for the estimation of amounts of aluminium between 0·3 and 1·0 mg. present in suspensions produced by adding potassium alum or other aluminium salts to *C. diphtheriae* culture filtrates has been described. The method depends on the precipitation of the aluminium with 8-hydroxyquinoline and the estimation of the amount of the latter contained in the precipitate.

I wish to express my thanks to Dr J. Trevan for his helpful interest, and to my assistant, C. Kennedy, for the care with which he has carried out a number of estimations during the course of this work.

REFERENCES.

Berg (1926). *Chem. Ztg.* **50**, 754.
Glenny and Barr (1931). *J. Path. Bact.* **34**, 131.
—— Pope, Waddington and Wallace (1926). *J. Path. Bact.* **29**, 38.
Myers, Mull and Morrison (1928). *J. Biol. Chem.* **78**, 595.
Pichler (1929). *Mikrochemie (Festschrift)*, 6.
Wallace (1927). *J. Path. Bact.* **30**, 667.

CCX. THE BODY FATS OF THE PIG.

I. INFLUENCE OF INGESTED FAT ON THE COMPONENT FATTY ACIDS.

By RAMKANTA BHATTACHARYA
and THOMAS PERCY HILDITCH.

From the Department of Industrial Chemistry, University of Liverpool.

(*Received October 16th, 1931.*)

An opportunity to study the component fatty acids present in the depôt fat of pigs, on similar lines to the recent examination of butter fats [Hilditch and Sleightholme, 1931] and of tallows [Banks and Hilditch, 1931], was afforded when Prof. J. C. Drummond and Captain Golding supplied us with specimens of the back fats and perinephric or leaf fats from three sets of pigs which had been fed on a control diet (i) alone or supplemented with (ii) a solid or (iii) a liquid fat.

The animals were from two litters and were fed on the experimental diet from the age of 3 months until 7 months, when they were slaughtered; the average weight at the commencement of the feeding tests was about 50 lbs., and that at death about 200 lbs.

Four pigs were fed solely on a control ration consisting of 60 % barley meal, 20 % flaked maize, 15 % soya bean meal and 5 % dried yeast (with 2 lbs. ground limestone per 100 lbs. mixture); four pigs received the control ration with 3 % of shea butter fat added; and five pigs received the control ration with 3 % of arachis oil added.

The shea butter fat was supplied through the Imperial Institute and, according to an analysis made in this laboratory, its component fatty acids were myristic (0·4 %), palmitic (8·5 %), stearic (35·9 %), oleic (49·9 %) and linoleic (5·3 %) acids. The arachis oil employed was not specifically examined, but the composition of arachis oil fatty acids is of the following order [Jamieson, Baughman and Brauns, 1921]: 6–8 % palmitic, 5–6 % stearic, 3–4 % arachidic, 3 % lignoceric, 53–61 % oleic, and 22–25 % linoleic acid. The control ration itself was estimated to contain 1·5 % of fat and 12 % of proteins.

The specimens of back fat supplied were divided in each case into two parts along the approximately central "streak" or seam of connective tissue, and the component acids of the fats in the "outer" (*i.e.* nearer the skin)

layer and the "inner" layer were determined separately: differences in composition parallel with the noticeable differences in softness of the inner and outer layers were observed in all three sets of fats. Of the perinephric fats, those from the control ration animals and from the control ration with shea fat pigs were slightly infected by moulds when received; changes due to the onset of rancidity may affect to some extent the accuracy of the linoleic acid figures in these instances.

The quantity of material available was restricted, especially as regards the back fats; but, fortunately, the mixture of fatty acids in lard is comparatively simple and we believe that the data presented below are of the usual order of accuracy for fractionation analyses. It has not, of course, been practicable, with these small quantities of mixed fatty acids, to seek for evidence of the possible presence of minor amounts of the higher saturated acids characteristic of arachis oil in the fats from pigs in the diet of which 3 % of this oil had been included. We also considered it unnecessary, for the immediate purpose, to determine the small amount (up to 0·4 %) of highly unsaturated arachidonic acid which Brown and Deck [1930] have shown to be present in normal lards.

The fats were separated from tissue, *etc.*, by extraction with hot acetone, the final traces of which were removed by heating in a vacuum at 100°. The saponification equivalent, iodine value and refractive index (at 60°) of each fat, and the setting-point of the corresponding mixed fatty acids, are collected in Table I.

Table I.

Feed	Body fat	Saponification equivalent	Iodine value	$n_D^{60°}$	Mixed acids. Setting-point
Control ration	Outer back	286·1	62·6	1·4512	38·8°
" "	Inner back	286·7	55·0	1·4507	41·8°
" "	Leaf (perinephric)	286·0	45·7	1·4500	44·2°
Control + 3 % shea fat	Outer back	286·1	65·2	1·4519	38·5°
" "	Inner back	285·4	57·0	1·4510	41·0°
" "	Leaf	284·7	43·9	1·4503	45·0°
Control + 3 % arachis oil	Outer back	283·7	68·9	1·4520	37·0°
" "	Inner back	285·8	61·7	1·4508	40·1°
" "	Leaf	286·7	55·1	1·4501	44·0°

The component fatty acids were estimated in each case by preliminary separation into "solid" and "liquid" acids by crystallisation of the lead salts from 95 % alcohol, followed by fractional vacuum distillation of the methyl esters of each group; the details of the procedure were the same as in the case of the corresponding analyses of beef tallows [Banks and Hilditch, 1931], except that the amount of esters handled precluded in most cases any refractionation of the primary ester fractions. Since the tabulation of the complete fractionation data occupies excessive space, the final results only are summarised in Table II: the figures in brackets indicate the weights of mixed fatty acids employed in each fractionation analysis.

Table II. *Component fatty acid analyses of the nine pig fats.*

Acid	Solid acids	Liquid acids	Total	Fatty acids (excluding unsaponifiable matter) % (wt)	% (mols.)
	Feed: control only. Outer back fat (29·7 g.).				
	(38·1 %)	(61·9 %)			
Myristic	—	2·03	2·03	2·0	2·4
Palmitic	24·51	—	24·51	24·6	26·3
Stearic	10·58	—	10·58	10·6	10·2
Oleic	3·01	50·15	53·16	53·3	51·8
Linoleic	—	9·46	9·46	9·5	9·3
Unsaponifiable	—	0·26	0·26	—	—
	Feed: control only. Inner back fat (88·3 g.).				
	(45·7 %)	(54·3 %)			
Myristic	—	1·40	1·40	1·4	1·7
Palmitic	29·51	—	29·51	29·6	31·6
Stearic	13·94	—	13·94	13·9	13·4
Oleic	2·25	44·80	47·05	47·2	45·6
Linoleic	—	7·87	7·87	7·9	7·7
Unsaponifiable	—	0·23	0·23	—	—
	Feed: control only. Leaf (perinephric) fat (97·1 g.).				
	(51·0 %)	(49·0 %)			
Myristic	—	3·65	3·65	3·6	4·4
Palmitic	28·40	—	28·40	28·5	30·2
Stearic	21·33	—	21·33	21·4	20·5
Oleic	1·27	39·95	41·22	41·3	39·9
Linoleic	—	5·18	5·18	5·2	5·0
Unsaponifiable	—	0·22	0·22	—	—
	Feed: control + 3 % shea fat. Outer back fat (48·75 g.).				
	(37·3 %)	(62·7 %)			
Myristic	—	3·64	3·64	3·7	4·4
Palmitic	24·93	1·05	25·98	26·1	27·8
Stearic	9·42	—	9·42	9·5	9·1
Oleic	2·95	47·15	50·10	50·3	48·6
Linoleic	—	10·37	10·37	10·4	10·1
Unsaponifiable	—	0·49	0·49	—	—
	Feed: control + 3 % shea fat. Inner back fat (30·7 g.).				
	(41·5 %)	(58·5 %)			
Myristic	1·09	3·57	4·66	4·7	5·5
Palmitic	26·50	—	26·50	26·6	28·2
Stearic	10·01	—	10·01	10·0	9·6
Oleic	3·72	47·22	50·94	51·0	49·2
Linoleic	—	7·71	7·71	7·7	7·5
Unsaponifiable	0·18	—	0·18	—	—
	Feed: control + 3 % shea fat. Leaf (perinephric) fat (73·2 g.).				
	(50·1 %)	(49·9 %)			
Myristic	0·07	2·75	2·82	2·8	3·3
Palmitic	30·84	—	30·84	30·8	32·7
Stearic	17·87	—	17·87	17·9	17·2
Oleic	1·32	43·57	44·89	44·9	43·3
Linoleic	—	3·58	3·58	3·6	3·5
Unsaponifiable	—	—	—	—	—
	Feed: control + 3 % arachis oil. Outer back fat (50·2 g.).				
	(37·1 %)	(62·9 %)			
Myristic	—	1·53	1·53	1·5	1·9
Palmitic	23·47	0·69	24·16	24·3	26·0
Stearic	8·95	—	8·95	9·0	8·7
Oleic	4·68	47·20	51·88	52·2	50·7
Linoleic	—	12·93	12·93	13·0	12·7
Unsaponifiable	—	0·55	0·55	—	—

Table II (*cont.*)

Feed: control + 3 % arachis oil. Inner back fat (74·0 g.).					
	(41·7 %)	(58·3 %)			
Myristic	0·16	1·37	1·53	1·5	1·8
Palmitic	24·38	—	24·38	24·5	26·2
Stearic	13·67	—	13·67	13·7	13·3
Oleic	3·49	45·55	49·04	49·3	47·9
Linoleic	—	10·98	10·98	11·0	10·8
Unsaponifiable	—	0·40	0·40	—	—
Feed: control + 3 % arachis oil. Leaf (perinephric) fat (117·7 g.).					
	(45·3 %)	(54·7 %)			
Myristic	—	1·70	1·70	1·7	2·0
Palmitic	24·98	0·58	25·56	25·6	27·4
Stearic	18·16	—	18·16	18·2	17·5
Oleic	2·16	42·03	44·19	44·2	43·0
Linoleic	—	10·29	10·29	10·3	10·1
Unsaponifiable	—	0·10	0·10	—	—

Discussion.

It is desirable, as in previous communications, to consider the constitution of these various fatty acid mixtures from the point of view of molecular proportion rather than weight percentage. In Table III the molar characteristics of the mixed fatty acids are arranged primarily so as to display the variations in the corresponding depôt fats due to the different diets: section (i) shows the molar distribution of the individual acids, and in section (ii) column I gives the total molar percentage of saturated acids, column II that of the combined stearic, oleic and linoleic acids, column III that of stearic acid, and column IV the molar proportion of linoleic in the unsaturated acids present.

Table III.

(i) *Molar distribution of individual acids.*

Fat	Feed	Myristic	Palmitic	Stearic	Oleic	Linoleic
Outer back	Control	2·4	26·3	10·2	51·8	9·3
,,	,, +3 % shea	4·4	27·8	9·1	48·6	10·1
,,	,, +3 % arachis	1·9	26·0	8·7	50·7	12·7
Inner back	Control	1·7	31·6	13·4	45·6	7·7
,,	,, +3 % shea	5·5	28·2	9·6	49·2	7·5
,,	,, +3 % arachis	1·8	26·2	13·3	47·9	10·8
Leaf (perinephric)	Control	4·4	30·2	20·5	39·9	5·0*
,,	,, +3 % shea	3·3	32·7	17·2	43·3	3·5*
,,	,, +3 % arachis	2·0	27·4	17·5	43·0	10·1

(ii) *Molar percentages of total saturated acids (I), total C_{18} acids (II), stearic acid (III) and proportion of linoleic acid in the unsaturated acids (IV).*

Fat	Feed	I	II	III	IV
Outer back	Control	38·9	71·3	10·2	15·1
,,	,, +3 % shea	41·3	67·8	9·1	17·1
,,	,, +3 % arachis	36·6	72·1	8·7	20·0
Inner back	Control	46·7	67·7	13·4	14·3
,,	,, +3 % shea	43·3	66·3	9·6	13·1
,,	,, +3 % arachis	41·3	72·0	13·3	18·3
Leaf (perinephric)	Control	55·1	65·4	20·5	11·5*
,,	,, +3 % shea	53·2	64·0	17·2	7·5*
,,	,, +3 % arachis	46·9	70·6	17·5	18·9

* These figures may be slightly low, owing to rancidity.

The data in Table III show certain similarities and certain differences when compared with the corresponding figures for tallows [Banks and Hilditch, 1931, Tables VIII, IX, X]. In the depôt fats of both cattle and pigs, palmitic acid is present in relatively the most constant proportions (25–33 mols. %). Furthermore, in both classes, variation in the proportion of saturated to unsaturated acids as a whole (column I) is not accompanied by anything approaching the same variation in the total content of C_{18} acids (or, therefore, of palmitic and myristic acids): whilst the extreme limits of total saturated acid content lie between 36·6 and 55·1 mols. %, those of the total C_{18} acid group range only from 64·0 to 72·1 mols. %. Thus, both in the pig body fats and in the tallows, variation in the amount of unsaturated acids is mainly compensated for by corresponding changes in the stearic acid content.

Differences between the pig and cattle depôt fats consist chiefly in the lower content of myristic acid in most of the former class and especially in the higher proportion of linoleic acid present. Thus, whilst in tallows the relative amount of linoleic acid rarely exceeds 10 %, and is frequently only about 5 %, of the combined oleic and linoleic acids, the corresponding proportions in the pig fats studied range from 10 % or a little less to as much as 20 %: also, the proportion of linoleic acid (i) clearly increases with increase of unsaturation in fatty oil forming part of the diet and (ii) also increases (at all events when unsaturated fatty oils are absent from the diet) from the perinephric fat to the outer layers of the back fat.

The general effects produced by the inclusion of either a hard or a soft fat in the diet may next be considered. The shea butter fat used contained 45 mols. of saturated acids with 55 mols. of unsaturated acids, that is, it was less saturated than the inner back or leaf fats from the control-fed pigs. Its ingestion caused little alteration in the total saturation of the depôt fats, but the total C_{18} acid content was somewhat lower than in the corresponding control fats, so that the palmitic and (especially) the myristic acid contents increased (although the relative proportion of palmitic (with myristic) to stearic acid in the shea fat itself is far below the corresponding ratio in any of the pig fats).

The arachis oil feed had a very marked influence upon the total unsaturation of the fats, which increased in each case (the alteration being greatest in the leaf fat and least in the outer back layer), and also upon the relative proportions of linoleic and oleic acid. The unsaturated acids contained about 20 % of linoleic acid, this proportion being approached in the leaf and inner back fats as well as in the outer back layer. On the other hand, increase in unsaturation in the arachis oil feed experiments was remarkably closely balanced by diminution in stearic acid—the molar percentages of palmitic (with myristic acid) being 29·4, 28·0 and 27·9 in the leaf, inner and outer back fats respectively.

It is noticeable that alteration in the diet had relatively less effect on the composition of the outer layer of back fat than on that of the inner layer or

of the leaf. The analyses, also, point to an essential difference between the average composition of the fat between the "streak" and the skin, and that of the fat on the inner side of the "streak." The former is definitely the most unsaturated and also contains the highest proportion of linoleic to oleic acid: the latter approximates more nearly in composition to its corresponding perinephric fat than to the outer back fat, although it is in general less saturated than perinephric fat. The data may perhaps be summed up in the statement that, whilst ingested fat has a marked effect on the character of the leaf and inner back fats, that of the outer layer of back fat is, to a considerable degree, similar in spite of such variation in diet as is represented by the present series of feeding experiments.

The relative constancy of the outer back fat in composition may, of course, be determined by adjustment of the fat nearest the skin to a more or less constant consistency adapted to the average temperature conditions of the external atmosphere. Henriques and Hansen [1901] give details of the melting-points and iodine values of body fats similar to our figures and conclude that these characteristics are mainly determined by the external temperature. It is desirable, in this connection, to ascertain whether the layers of fat on either side of the "streak" are homogeneous, or whether there is a progressive alteration as the outer skin is approached: it is hoped in due course to examine the whole of the back fat from a single animal, divided into two or more sections on each side of the "streak," in order to settle this point.

Some further observations must be offered with reference to the tendency to constant molar content of the total C_{18} acids (or, alternatively, of the saturated acids other than stearic), which has now been remarked in the case of the component fatty acids of butter-fats, tallows and lards. In the tallows it was pointed out that the available data showed the possible existence of two classes, one containing about 62 mols. %, and the other about 70 mols. %, of total C_{18} acids. The present series of analyses takes us a little further: the range of this figure, for the pig fats now studied, is from 64·0 to 72·1 mols. % of C_{18} acids, and, broadly speaking, fats with a high content of oleic and linoleic acids have values towards the upper limit for the total C_{18} acids, and conversely. In other words, although the general constancy of the total C_{18} acid content is marked in comparison with the variation in total unsaturated acid content, yet, within this generalisation, it seems clear that increase of oleic and linoleic acids is not completely balanced by diminution in stearic acid, but that, to a subordinate extent, the total C_{18} acid content is swollen and that of the other saturated acids, palmitic and myristic, somewhat diminished.

The results given in this paper thus support the tentative suggestions offered at the conclusion of the discussion of the glyceride structure of tallows [Banks and Hilditch, 1931], namely:

(i) that ingested vegetable fats are utilised by disposal of excess of linoleic and oleic acids by oxidation, leaving a residue of palmitic (with minor quan-

tities of myristic and stearic), oleic and linoleic acids more or less adapted to the requirements of the particular storage fat (conversion of carbohydrates, *etc.*, to fat is of course another, but separate, source of depôt fats);

(ii) that the stearic acid content of body-fats, which is relatively much greater than that of the fats present in a vegetable diet, is secured by processes involving "saturation" of unsaturated acids.

It further appears that, in the case of the pig, the amount of linoleic acid tolerated in the storage fat is considerably greater than in tallows, and, moreover, that it is in some degree related to that ingested in the diet; whilst inspection of the data in Table III shows that there is some evidence that, if stearic acid results from saturation of unsaturated acids, in the pig fats it is almost entirely oleic acid, and not linoleic acid, which diminishes as the content of stearic acid rises[1].

These views accord in general, if not in all details, with those at which Ellis and co-workers have arrived in the course of their recent studies on soft pork. The percentage of each individual component acid was determined in the cases of relatively few of the numerous samples of fat dealt with by these investigators, but we have found it of great interest to convert such data, where available, into molar proportions for comparison with our own results.

In a study of the influence of a ration low in fat upon the composition of the body fat of hogs, Ellis and Zeller [1930] quote individual acid figures for the composite body fat of hogs of varying age and weight. These are reproduced, in the form of molar proportions, in Table IV.

Table IV. *Composite body fats of hogs of varying age, fed on rations low in fat* [*Ellis and Zeller, 1930*].

(i) *Molar distribution of individual acids.*

Hog No.	Age (days)	Live wt. (lbs.)	Fat I. V.	Myristic	Palmitic	Stearic	Oleic	Linoleic
5	110	73	61·2	1·4	27·4	8·2	56·5	6·5
7	134	110	57·4	0·9	29·9	8·7	56·0	4·5
11	246	243	53·3	1·3	28·0	11·1	58·8	0·8
13	257	282	55·1	0·9	27·2	10·7	59·9	1·3

(ii) *Molar percentages of total saturated acids* (*I*), *total* C_{18} *acids* (*II*), *stearic acid* (*III*) *and proportion of linoleic acid in the unsaturated acids* (*IV*).

Hog No.	I	II	III	IV
5	37·0	71·2	8·2	10·3
7	39·5	69·2	8·7	7·4
11	40·4	70·7	11·1	1·4
13	38·8	71·9	10·7	2·2

The fat content of the diet in this series did not exceed 0·5 %, whereas in the control feed of the pigs whose body fats we have analysed there was

[1] Ellis and Harkins [1925] express the opinion that linoleic acid is not so essential a factor in adipose tissue as is oleic acid, since the amount appears to be controlled by the amount furnished in the feed; whereas deposited linoleic acid is usually less than the total amount ingested, the amounts of total saturated acids and of oleic acid are greatly in excess of the supply in the feed.

1·5 % of fat, and the pigs were killed when about 210 days old and at an average live weight of about 200 lbs. The component fatty acids of the outer back fat of pigs fed on our control diet are almost identical with the mean of Ellis and Zeller's figures for hogs 7 and 11, and it will be noticed that the nearly constant total C_{18} acid molar content in Table IV (69·2–71·9 %) accords exactly with our observations. The chief difference is in the proportion of linoleic acid, which is lower than in our experience and finally diminishes to 2 % or less of the unsaturated acids; it may well be, therefore, as Ellis and Zeller suggest, that the linoleic acid in pig depôt fat is derived exclusively from assimilated vegetable linoleic acid.

Ellis and Isbell [1926] investigated the influence of ingested fats in a somewhat drastic manner by feeding pigs (*a*) on brewer's rice, tankage and grass, (*b*) on corn, skim milk and grass, (*c*) on soya beans alone and (*d*) on arachis nuts alone. Their figures for component fatty acids (reduced to molar proportions as in Table IV) are given in Table V.

Table V. *Body fats of hogs fed on various fatty diets* [*Ellis and Isbell, 1926*].

(i) *Molar distribution of fatty acids.*

Feed	Fat from	I. V.	Myristic	Palmitic	Stearic	Arachidic	Oleic	Linoleic	Linolenic
Brewer's rice, *etc.*	Back	52·6	2·1	28·2	11·7	—	56·8	1·2	—
Corn, *etc.*	Meat	58·8	0·8	27·0	12·3	—	53·0	6·9	—
Soya beans alone	Back	90·7	0·9	18·7	9·2	—	39·6	31·4	0·2
,,	Back	100·6	0·4	15·3	7·7	—	38·2	37·9	0·5
Arachis									
(Virginian)	Meat	84·1	0·5	16·8	7·3	0·2	55·9	19·3	—
(Spanish)	Meat	91·8	0·1	11·3	4·8	0·3	63·9	19·6	—

(ii) *Molar percentages of total saturated acids (I), total C_{18} acids (II), stearic acid (III) and proportion of linoleic acid in the unsaturated acids (IV).*

Feed	Fat from	I. V.	I	II	III	IV
Brewer's rice, *etc.*	Back	52·6	42·0	69·7	11·7	2·1
Corn, *etc.*	Meat	58·8	40·1	72·2	12·3	11·5
Soya beans alone	Back	90·7	28·8	80·4	9·2	44·3
,,	Back	100·6	23·4	84·3	7·7	49·8
Arachis (Virginian)	Meat	84·1	24·8	82·5	7·3	25·7
,, (Spanish)	Meat	91·8	16·5	88·3	4·8	23·5

This remarkable series shows that, whilst a balanced ration containing not more than 5 % of vegetable fat leads to body fats of exactly the same type as those previously discussed (Tables III and IV), a ration of vegetable seeds alone leads to the following effects: (*a*) the complete breakdown of any constancy in the total C_{18} acids, and consequently considerable diminution in the molar content of palmitic acid, (*b*) increase in the linoleic acid to proportions approaching those in the ingested fats and (*c*) failure to produce stearic acid to compensate for the reduction in palmitic acid or, indeed, to the normal amount of 10–20 mols. % observed with an ordinary balanced diet. Ellis and Isbell attach some significance to the approximate ratio of

2 : 1 for palmitic and stearic acids in these extreme cases, but examination of the wider range of data in Tables III and IV proves that this ratio may range from over 3 : 1 to below 1·5 : 1; in our opinion these relative proportions are not so material as the general relationships between the total C_{18} acid content and that of palmitic (with myristic) acids, coupled with the balance, within the C_{18} group, between stearic and the unsaturated acids (especially oleic acid).

The concentration of fat in the diet beyond which the tendency to constant proportions of palmitic (with myristic) and of total C_{18} acids clearly disappears is further established by the recent experiments of Ellis, Rothwell and Pool [1931] in which pigs were fed on a basal diet of hominy, tankage, alfalfa meal and mineral mixture (containing less than 1 % fat) supplemented by varying amounts of cotton-seed oil, the component fatty acids of which are myristic (0·5 %), palmitic (22 %), stearic (2 %), oleic (30·5 %) and linoleic (45 %). Their results are reduced to molar proportions in Table VI.

Table VI. *Composite back fats of hogs fed on varying rations of cotton-seed oil* [*Ellis, Rothwell and Pool, 1931*].

(i) *Molar distribution of fatty acids.*

Feed	Fat I. V.	Myristic	Palmitic	Stearic	Oleic	Linoleic
Basal diet only	60·6	2·0	27·3	13·2	48·8	8·7
„ + 4 % C.S.O.	60·5	1·4	26·8	20·4	38·4	13·0
„ + 8 % „	64·4	1·0	23·5	22·6	35·0	17·9
„ +12 % „	77·4	1·3	15·0	25·9	31·3	26·5

(ii) *Molar percentages of total saturated acids (I), total C_{18} acids (II), stearic acid (III) and proportion of linoleic acid in the unsaturated acids (IV).*

Feed	Fat I. V.	I	II	III	IV
Basal diet only	60·6	42·5	70·7	13·2	15·2
„ + 4 % C.S.O.	60·5	48·6	71·8	20·4	25·1
„ + 8 % „	64·4	47·1	75·5	22·6	33·8
„ +12 % „	77·4	42·2	83·7	25·9	45·8

Thus, at some point between 4 and 8 % of added fat in the diet, the total C_{18} acid content ceases to be approximately constant and rises considerably, although in this instance the molar content of the C_{18} acids in the ingested cotton-seed oil itself is only 75·8 %. (It is noteworthy that, with the larger amounts of cotton-seed oil, the stearic acid content of the pig fats continued to augment, in contrast to the observations with animals fed on soya beans or arachis nuts; the parallel differences in the diet, compared with that of these oil seeds alone, are (i) the presence throughout of a substantial, if reduced, proportion of basal meat ration and (ii) the presence in the cotton-seed oil acids of about 24 mols. % of palmitic acid.)

The definite approach to a constant balance between palmitic (with myristic) acids and the total C_{18} acids which we have established in the cases of the body fats of pigs and cattle and of the milk fat of cows fed on what

may be termed a "normal" diet, coupled with the breakdown of this balance in the pig fats examined by Ellis and collaborators when more than a certain proportion of added fat was present in the diet, may be regarded in another way: a molar content of 30 % palmitic (with myristic) acid is attained in the pig depôt fats only when the quantity of ingested fat in the diet is small (not exceeding about 5 %). This is additional, if indirect, evidence that a considerable proportion of the normal depôt fat must be derived by synthesis in the animal from the non-fatty components of the diet, rather than by direct assimilation of fat. This deduction appears to be of at least equal importance, as regards the normal animal metabolic processes, to the more obvious one that, with an excessive fatty diet deficient in carbohydrates, *etc.*, the pig is capable, *faut de mieux*, of utilising directly much of the ingested fats for storage.

Lack of material, as stated previously, prevented us from including a study of the glyceride structure of the nine fats which we have examined. A full examination of the component fatty acids and glyceride structure of the corresponding fats from a single pig fed on a controlled diet is, however, nearly complete, and the additional data which will then be available will enable us to offer a possible general explanation of some of the features connected with the elaboration of animal fats and of the fundamental differences in glyceride structure between these and the vegetable seed fats.

Summary.

The component fatty acids of the perinephric fats and the outer and inner layers of the back fats from pigs fed on a basic diet (i) alone or supplemented by 3 % of (ii) shea butter or (iii) arachis oil have been determined. The general relationships which were recently observed amongst the various classes of fatty acids of tallows are found to be present also in the pig body fats, more especially a tendency to approximate constancy in the molar content of C_{18} acids in spite of variation in the total proportion of saturated to unsaturated acids; increase in saturation is mainly produced by increase of stearic acid and corresponding diminution in oleic acid.

The composition of the outer layer of back fat differed in each case from that of the inner layer, the latter being intermediate in type between the former and the perinephric fat, which it more closely resembled. The outer layer of back fat was the most unsaturated, but the differences between the three depôt fats were much reduced when the diet of the animals included arachis oil.

As regards the influence of ingested fat in the diet, the body fats from animals whose feed included the relatively saturated shea butter were not greatly different from those of the control-fed animals, but presence of arachis oil in the diet led definitely to increase of unsaturation in the stored fat and also to increase in the proportion of linoleic acid in the unsaturated acids.

Consideration of the experimental data in conjunction with the results of Ellis and collaborators on the feeding of pigs with rations containing high proportions of vegetable fat confirms our previous conclusions as to the general rules governing the composition of depôt fat of animals fed on a balanced diet, and shows clearly the amount of added fat (beyond about 5–8 % of the diet) which leads to the breakdown of these characteristics. Further, the data as a whole prove that much of the normal depôt fat of the pig must be derived from carbohydrate or other non-fatty part of the feed.

We are most grateful to Prof. Drummond, Captain Golding and the Director of the Imperial Institute for having assisted us to obtain the material for these observations; and we would also tender our thanks to the Government of the Central Provinces of India for a scholarship held by one of us (R. B.) during the course of the work.

REFERENCES.

Banks and Hilditch (1931). *Biochem. J.* **25**, 1168.
Brown and Deck (1930). *J. Amer. Chem. Soc.* **52**, 1135.
Ellis and Harkins (1925). *J. Biol. Chem.* **66**, 101.
—— and Isbell (1926). *J. Biol. Chem.* **69**, 239.
—— Rothwell and Pool (1931). *J. Biol. Chem.* **92**, 385.
—— and Zeller (1930). *J. Biol. Chem.* **89**, 185.
Henriques and Hansen (1901). *Skand. Arch. Physiol.* **11**, 151.
Hilditch and Sleightholme (1931). *Biochem. J.* **25**, 507.
Jamieson, Baughman and Brauns (1921). *J. Amer. Chem. Soc.* **43**, 1372.

CCXI. THE LIBERATION OF ELEMENTARY NITROGEN BY BACTERIA.

By NEWTON WOOD BARRITT.

From the General Microbiology Department, Rothamsted Experimental Station.

(*Received October 19th, 1931.*)

The loss of nitrogen during the putrefaction of organic matter has been the subject of numerous investigations during the past 100 years. In a review of the literature of the subject by Jensen [1906] there appears to be a difference of opinion as to the source of the nitrogen loss and of the conditions which give rise to it. Thus Reiset [1856], Dehérain and Dupont [1898] and Gibson [1895] found that nitrogen was a definite end-product in the putrefaction of meat. Lawes, Gilbert and Pugh [1861] also found that nitrogen was evolved in the aerobic decomposition of vegetable matter, and Schloesing [1889, 1] tried to confirm this result but not very successfully.

This conclusion was not supported by the work of Hüfner [1876], Kellner and Yoshi [1888], Tacke [1887] and Immendorff [1892]. These authors found that the liberation of free nitrogen only occurred in the presence of nitrites or nitrates. Jensen is of opinion that the results of the earlier workers are not reliable owing to faulty analytical methods.

Löhnis [1926] states that a loss of nitrogen from organic matter occurs only in the presence of air, and that it has not yet been proved that free nitrogen is derived from the amino-group or ammonia. He suggests the possibility of a liberation of nitrogen by the purely chemical interaction between amines or NH_3 and nitrous acid thus:

$$R.NH_2 + HNO_2 = R.OH + N_2 + H_2O$$
$$NH_3 \quad + HNO_2 = N_2 + 2H_2O$$

This possibility was first put forward by Dietzell [1882], though the reaction was first discovered by Piria [1849]. Fowler and Kotwal [1924] found it to be ineffective except in the presence of mineral acid. Grimbert [1899], however, suggested that a liberation of amino-nitrogen may occur in a nitrate-broth medium without the development of acidity or perhaps of only local and temporary acidity.

Russell [1927] agrees with Jensen as to the unreliability of the analytical methods used by the earlier workers but considers the results of Lawes, Gilbert and Pugh [1861] and of Pfeiffer *et al.* [1897] as sufficient evidence of the possibility of nitrogen being a final product of protein breakdown. In collaboration with Richards [1917], however, he showed that no nitrogen

was evolved in the decomposition of dung under completely aerobic or completely anaerobic conditions but only under a combination of the two conditions. He accounted for the result of Lawes, Gilbert and Pugh and others by the formation under anaerobic conditions of unstable intermediate products which evolve nitrogen on contact with air. Waksman [1927] in his review of the literature does not refer to the possibility of the liberation of free nitrogen without denitrification.

Since the work of Lawes, Gilbert and Pugh, and of Pfeiffer on the liberation of nitrogen from organic matter by oxidation, three authors have recorded the same phenomenon under anaerobic conditions. Wood and Willcox [1897] obtained considerable quantities (150 cc.) of nitrogen from a peptone-glucose medium inoculated with a pure culture of a bacillus isolated from fermenting bran used in the bating of hides. Unfortunately this experiment is not above criticism since oxygen gas was also collected, which, as a product of anaerobic fermentation, suggests the leakage of air into the apparatus. Harden [1901], using a peptone-glucose medium inoculated with *B. coli*, obtained a small amount of nitrogen (7·5 cc.) but none in absence of glucose. The rôle of the sugar in promoting the evolution of nitrogen is somewhat obscure, since carbohydrates are known to have a depressing effect on ammonification [Doryland, 1916], which would presumably be an intermediate stage in the formation of free nitrogen. Bach and Sierp [1923], using sewage sludge, obtained considerable quantities of nitrogen during anaerobic (?) fermentation in the presence of large volumes of tap-water. As pointed out by Buswell and Neave [1927] the results are subject to a very large error from the daily exchange of 500 cc. of tap-water containing sufficient dissolved air to account for all the nitrogen collected.

Thus, the evidence amply bears out Pfeiffer's remarks that "there is scarcely another field of research in agricultural chemistry in which we encounter contradictions so numerous and so fully unexplained."

As Jensen points out, the position regarding the formation of free nitrogen by bacteria is complicated, not only as regards the source of the nitrogen but also as to the conditions which give rise to it.

Amidst the conflicting evidence of previous workers there is one process of the liberation of free nitrogen by bacteria that is beyond question, *viz.* denitrification. According to Mair [1908], Beijerinck and Minkman [1910] and Lloyd and Cranston [1930], nitrates are reduced in three stages: (1) nitrite, (2) hyponitrite, (3) free nitrogen. It follows therefore that in all attempts to prove the liberation of nitrogen by the oxidation of amines or ammonia it is essential to ensure the absence of nitrates, nitrites and hyponitrites. The presence of nitrifying bacteria during putrefaction has been well established by numerous workers. These organisms are now recognised as taking part in biological oxidation in symbiosis with heterotrophic organisms [Mair, 1908; Barritt, 1931].

Ammonification studies have been carried out by numerous workers in

recent years, and the production of ammonia is now regarded as an essential part of the bacterial oxidation of organic matter when the C/N ratio of the substrate is not too high [Marschal, 1893; Doryland, 1916]. The yield of ammonia is quantitative and no recent worker has recorded a loss of free nitrogen. The only certain products of the biological oxidation of ammonia are nitrite and nitrate, and, since a crude inoculation of soil organisms will oxidise ammonia quantitatively to nitrous or nitric acid [Schloesing, 1889, 2], there is no reason to suppose the existence in soil of organisms capable of oxidising ammonia to free nitrogen.

These observations are amply borne out by the work of Russell and Richards [1917] on the loss of nitrogen during the decomposition of manure, and by the work of the Royal Commission on Sewage Disposal [1908]. In both cases loss of nitrogen was only recorded in the presence of nitrates, which represent the balance of the nitrification process in excess of denitrification as stated above.

Since denitrification appears to be an essential part of the process, the following investigations were undertaken with a view to obtaining more precise data on this process and also on the possible interaction of amines and ammonia with nitrous acid, a subject which has received little attention from previous workers. The experiments of Fowler and Kotwal [1924] on this point involved small gasometric measurements (2 cc.) and are not at all convincing.

Experimental.

Culture solutions of 0·5 % concentration of the following substances were prepared with the addition of 0·05 % potassium phosphate, 0·03 % sodium chloride and 0·1 % potassium nitrate: urea, uric acid, glycine, alanine, tyrosine, ammonium acetate, ammonium sulphate, dextrose + ammonium sulphate, dextrose, acetic acid and alcohol. The distilled water used was previously inoculated by shaking each litre with 1 g. of Broadbalk soil. The initial p_H was adjusted to 6·5. 200 cc. of each culture were incubated at 25° in a 300 cc. stoppered bottle for 6 weeks. Determinations of organic *plus* ammonia nitrogen were made by the Kjeldahl method, nitrites by the Griess-Ilosvay method and nitrate by the pyrogallol colorimetric method.

During the incubation period the reaction of the culture solutions was tested daily. The rise in p_H due to ammonification of organic nitrogen and liberation of alkali by the denitrification of the potassium nitrite was corrected by the addition of solid KH_2PO_4, and the lowering of p_H due to glycolysis was corrected to some extent by the addition of calcium carbonate. In this way an average p_H of 6·5 was maintained.

The formation of nitrite occurred in all cultures within 24 hours and persisted until all the nitrate had disappeared. The amount of nitrite produced varied with the different solutions, the greatest amounts (0·013 %) being recorded in the solutions containing dextrose and alcohol. The nitrite also per-

sisted in these solutions throughout the whole period of 6 weeks. The most vigorous reduction occurred in the solutions of uric acid and amino-acids, in which cases the amount of nitrite never exceeded 0·003 % and completely disappeared within 24 days. The slower rate of reduction in the carbohydrate solution is probably due to the lower p_H owing to the buffering action of the calcium carbonate being arrested by a covering of bacterial growth.

The formation of organic acids from dextrose does not require oxygen and the oxidation of these acids is very much reduced at $p_H < 6$ [Cook and Alcock, 1931]. For this reason the nitrite is more slowly reduced and therefore persists in the carbohydrate solutions for a much longer period than in the solutions containing amino-compounds. The results of the nitrogen estimations are given in Table I.

Table I.

		C/N ratio	Initial N g. per 100 cc.		Final N after incubation			Loss of N	
	Substance		organic $N+NH_3$	as N_2O_5	organic $N+NH_3$	N_2O_3	as N_2O_5	organic $N+NH_3$	as N_2O_5
1	Ammonium sulphate	—	0·053	0·014	0·0531	Trace	0·014	Nil	Nil
2	Urea	0·43	0·233	0·014	0·235	Trace	0·014	Nil	Nil
3	Uric acid	1·07	0·167	0·014	0·166	—	—	0·001	0·014
4	Glycine	1·71	0·0933	0·014	0·0928	—	—	0·0005	0·014
5	Ammonium acetate	1·71	0·0530	0·014	0·0523	—	—	0·0007	0·014
6	Alanine	2·57	0·0777	0·014	0·0776	—	—	0·0001	0·014
7	Tyrosine	7·7	0·0379	0·014	0·0372	—	—	0·0007	0·014
8	Dextrose + $(NH_4)_2SO_4$	10·0	0·0127	0·014	0·0124	—	0·0136	0·0003	0·0004
9	Dextrose only	14·5	—	0·014	0·00501	—	—	(increase 0·00501)	0·009
10	Acetic acid	14·5	—	0·014	0·00093	0·004	0·009	(increase 0·00093)	0·0007
11	Alcohol	18·0	—	0·014	0·00125	—	—	(increase 0·00125)	0·0127
12	Glycine + dextrose	3·5	0·0933	0·014	0·0916	0·0025	—	(loss 0·0017)	0·0115

It will be seen that there is no significant loss of organic nitrogen or ammonia in any of the solutions except No. 12 (glycine + dextrose), in which case a loss of 0·0017 g. nitrogen occurs. Comparing this result with that of No. 4 (glycine alone) it appears that the addition of dextrose to culture No. 4 (glycine alone) has resulted in a loss of organic nitrogen.

In the case of cultures Nos. 1 and 2, ammonium sulphate and urea, there was also no loss of nitrate nitrogen. Ammonification of the urea occurred, but no further change.

In cultures Nos. 3, 4, 5, 6 and 7 (of low C/N ratio) denitrification was complete in 21 days, and although these were incubated for a further period of 21 days no further loss of nitrogen occurred. Therefore the organisms concerned in the liberation of nitrogen during the first 3 weeks were unable to liberate nitrogen from either ammonia or organic nitrogen.

The higher C/N ratios of cultures Nos. 9, 10 and 11 appear to be associated with a smaller loss of nitrate nitrogen and a slight increase in organic nitrogen due to synthesis.

The loss of nitrogen in culture No. 12 appears at first sight to confirm Harden's observation of the liberation of nitrogen from protein in the presence of sugar. In the above case however nitrites were present, in which respect it differs from Harden's unless nitrates or nitrites were present as an impurity. Considerable amounts of lactic acid were formed in Harden's culture with glucose and it is well known that nitrites may react with amino-acids in presence of organic acid (*e.g.* acetic acid in Van Slyke's method of estimating amino-N). We may therefore expect a loss of amino-nitrogen in the presence of organic acids and nitrite. If nitrite was present in Harden's culture, free nitrogen should also have been formed in the peptone solution without glucose by the denitrification process. In this case however the amount of nitrogen liberated would be exactly half that formed by the interaction of amines with nitrous acid. Possibly 3·5 cc. of nitrogen is not sufficient for measurement with any degree of accuracy [see Russell and Richards, 1917]. In the cases where nitrite is present at the end of the fermentation the recorded loss of organic nitrogen might have occurred by means of the interaction between amines and nitrous acid on adding sulphuric acid for the Kjeldahl digestion. The possible loss of nitrogen at this stage was tested by adding excess nitrite to a 0·5 % solution of glycine before the acid digestion. The final organic nitrogen was identical with that of the materials without the glycine, indicating complete decomposition of the amino-group. In culture No. 12 therefore the recorded loss of organic nitrogen may have occurred during analysis and not during the fermentation.

In order to test the possibility of this reaction occurring during acid fermentation sterile solutions of glycine and sodium nitrite were prepared and varying quantities of lactic acid added. The amino-nitrogen was determined by Sørensen's method of formaldehyde titration. The results obtained are given in Table II.

Table II.

			Initial p_H	cc. $N/10$ NaOH required
0·5 % glycine	+ 0·5 % $NaNO_2$		6·5	31·0
,,	,,	+ lactic acid	6·2	30·6 (12 hrs.)
,,	,,	,,	5·7	30·5 ,,
,,	,,	,,	4·8	30·5 ,,
,,	,,	,,	4·2	28·4 ,,
,,	,,	,,	3·8	25·8 (5 mins.)
				7·8 (12 hrs.)

These results show that the rate of decomposition of the amino-acid is considerably diminished above p_H 4. It was afterwards found that the p_H of the less acid solutions increased rapidly, so that an initial p_H of 4·2 became 5·2 in 10 minutes and 6·5 in 1 hour. This rise in p_H is probably due to the volatility of the nitrous acid. At $p_H < 5·0$ a rapid evolution of gas occurred only on shaking the flask, and no gas appeared to be evolved when the solution remained quiescent. This liability to supersaturation with nitrogen gas must invalidate the results obtained by gasometric methods unless accompanied

by vigorous fermentation. With a continuous production of organic acid such as occurs in the fermentation of carbohydrates a more effective interaction between the amino-acid and nitrites could occur and result in a greater loss of free nitrogen.

Under strongly aerobic conditions organic acids are oxidised to carbonic acid with a consequent rise in p_H of the medium but, since nitrous acid is also liberated from nitrites by carbon dioxide [Moore, 1904], it seemed desirable to repeat these experiments with carbonic acid in place of lactic acid. Solutions of carbonic acid were obtained from a sparklet-siphon, using distilled water and liquid CO_2. A solution of calcium bicarbonate was also used. The results obtained are as follows:

Formaldehyde titrations of 0·5 % glycine + 0·5 % nitrite

	p_H	cc. $N/10$ NaOH required
In water only	6·8	31·0
In supersaturated solutions of CO_2 at atmospheric pressure 12 hours	5·8	23·2
In saturated solution of CO_2 under pressure	5·8	20·5
In calcium bicarbonate solution	6·0–6·8	30·0

These results show that it is possible to obtain a loss of nitrogen by the interaction of glycine and nitrite in a solution supersaturated with carbon dioxide. It is unlikely that such conditions would occur in the decomposition of organic matter. In the culture solutions described above (Table I) the reaction was maintained at p_H 6·5 and no loss of amino-nitrogen occurred [*cf.* Grimbert, 1899].

From these results it must be concluded that a loss of organic nitrogen is possible during denitrification provided that organic acids and amino-compounds are simultaneously produced. This event is more likely to occur when readily available carbohydrates like dextrose are present. It would also be expected to occur in the absence of nitrates when anaerobic conditions are followed by aeration. In the presence of nitrifying organisms amino-acids may be regarded as the unstable compounds referred to by Russell and Richards [1917] as oxidising to elementary nitrogen on exposure to air.

The simultaneous production of ammonia by the oxidation of protein and of nitrite by the reduction of nitrate suggests the possibility of free nitrogen being formed by the spontaneous decomposition of ammonium nitrite. This has been suggested by both Löhnis and Waksman. It is well known that ammonium nitrite is readily decomposed on heating, but in dilute solution it appears to be quite stable. A 0·5 % solution accurately prepared by mixing molecular equivalents of ammonium sulphate and sodium nitrite was incubated at 25° for 30 days without loss of either ammonia or nitrite and without a trace of gas being produced. The same result was obtained when the solution was incubated with soil. It must therefore be concluded that the decomposition of ammonium nitrite is not a source of the liberation of free nitrogen by bacteria.

SUMMARY.

1. A review of the literature shows that much confusion exists regarding the liberation of nitrogen by bacteria. The evidence of some workers that it occurs under anaerobic conditions is cancelled by that of others who show that it occurs only under aerobic conditions.

2. It is pointed out that inaccuracies in analytical methods and insufficient attention to the occurrence of nitrates or nitrification is responsible for the confusion as to the nature of the process.

3. Experiments are described which confirm the results of several previous workers that

(*a*) free nitrogen is liberated by soil bacteria during denitrification;

(*b*) there is no evolution of nitrogen from organic compounds under anaerobic conditions in the absence of oxidised nitrogen, nor from oxidised nitrogen in the absence of oxidisable organic compounds.

4. The reduction of nitrates and nitrites is effected most rapidly in the presence of compounds of low C/N ratio, and the formation of free nitrogen is strictly confined to the reduction of oxidised nitrogen provided the reaction of the medium does not fall below p_H 6·5.

5. The production of free nitrogen from organic compounds does not occur by bacterial action but only indirectly by the interaction between free nitrous acid and amino-compounds, both of which may be produced simultaneously by bacterial agency.

6. Free nitrous acid is liberated from nitrites by organic acids and carbonic acid, but does not effect the decomposition of amino-acids unless the p_H of the medium is $< 6{\cdot}0$.

7. The presence in a culture of readily available carbohydrates may result in a loss of free nitrogen from organic matter by the indirect process referred to in 5.

8. In Kjeldahl determinations of solutions of organic nitrogen the presence of nitrite results in a loss of amino-nitrogen during the acid digestion.

9. There is no liberation of free nitrogen by the decomposition of ammonium nitrite in culture solutions at ordinary temperatures.

The writer is much indebted to Mr R. G. Warren of the Chemical Department, for much useful advice and for the control of the Kjeldahl determinations, and also to Dr L. de Telegdy Kovato of Budapest (late of this department) for help in the discussion of the literature.

REFERENCES.

Bach and Sierp (1923). *Centr. Bakt. Par.* II, **58**, 401.
Barritt (1931). *Biochem. J.* **25**, 1419.
Beijerinck and Minkman (1910). *Centr. Bakt. Par.* II, **25**, 30.
Buswell and Neave (1927). *Soil Sci.* **24**, 285.
Cook and Alcock (1931). *Biochem. J.* **25**, 523.
Dehérain and Dupont (1898). *Ann. Agron.* **25**, 401.
Dietzell (1882). *Biederm. Centr. Agrik. Chem.* **11**, 417.
Doryland (1916). *N. Dakota Agric. Exp. Sta. Bull.* 116.
Fowler and Kotwal (1924). *J. Indian Inst. Sci.* **7**, 29.
Gibson (1895). *Wollnys Forschungen*, **18**, 106.
Grimbert (1899). *Ann. Inst. Pasteur*, **13**, 67.
Harden (1901). *J. Chem. Soc.* **79**, 612.
Hüfner (1876). *J. prakt. Chem.* **13**, 292.
Immendorf (1892). *Landw. Jahrb.* **21**, 281.
Jensen (1906). Lafar's Handbuch der Tech. Mykologie, III, 190. (Geb. Borntraeger, Berlin.)
Kellner and Yoshi (1888). *Z. physiol. Chem.* **12**, 95.
Lawes, Gilbert and Pugh (1861). *Phil. Trans. Roy. Soc. Lond.* 497.
Lloyd (1931). *J. Roy. Tech. Coll. Glasgow*, 530.
—— and Cranston (1930). *Biochem. J.* **24**, 529.
Löhnis (1926). Vorles. über Landw. Bakt. (Gustav Fischer, Berlin.)
Mair (1908). *J. Hygiene*, **7**, 609.
Marschal (1893). *Bull. Acad. Roy. Belgique* (3), **25**, 727.
Moore (1904). *J. Amer. Chem. Soc.* **26**, 959.
Pfeiffer, Franke, Gotze and Thurmann (1897). *Landw. Versuchs. Stat.* **48**, 189.
Piria (1849). See Cain's Chemistry and technology of the diazo-compounds, p. 1. (London.)
Reiset (1856). *Compt. Rend. Acad. Sci.* Vol. **42**.
Royal Commission on Sewage Disposal, 5th Report (1908), App. 6.
Russell (1927). Soil conditions and plant growth, p. 263. (London.)
—— and Richards (1917). *J. Agric. Sci.* **8**, 495.
Schloesing (1889, 1). *Compt. Rend. Acad. Sci.* **108**, 205.
—— (1889, 2). *Compt. Rend. Acad. Sci.* **109**, 423.
Tacke (1887). *Landw. Jahrb.* **16**, 917.
Waksman (1927). Principles of soil microbiology. (Ballière Tindall & Cox, London.)
Wood and Willcox (1897). *J. Soc. Chem. Ind.* **16**, 510.

CCXII. THE CHEMISTRY OF COOKING.

I. THE CHEMICAL CHANGES OF CARBOHYDRATES IN THE SWEET POTATO ACCORDING TO VARIOUS METHODS OF COOKING[1].

By OSAMU SINODA, SUZU KODERA and CHIYO OYA.

From the Laboratory of Household Chemistry, St Agnes' School for Girls, Kyoto.

(*Received October 21st, 1931.*)

Chemical analyses of the raw materials of our common food substances have already been made by various authors, but until now the changes of chemical composition during cooking have been little understood in spite of their evident importance from the point of view of nutritive value. The present paper represents a study of the chemical changes in the carbohydrates of the sweet potato brought about by various methods of cooking.

Method of cooking.

As the fundamental types of cooking, the authors took the following three procedures, boiling, steaming and baking. The conditions of the experiment are tabulated below. Materials were chopped and cooked just as they are prepared for the table, only without salt or any spices.

Table I. *Conditions of experiment.*

Exp. No.	A	B	C	D	E	F	G
Method of cooking	Boiling	Boiling	Boiling	Steaming	Steaming	Baking	Baking
Origin of the sample	Takatsuki Osaka-fu	Shinden Kyoto-fu	Do.	Yagi Hyogo-ken	Koma Kyoto-fu	Yagi Hyogo-ken	Koma Kyoto-fu
Weight (g.)	134	255	317	217	174	187	549
Water (cc.)	200	500	400	300	—	—	—
Added material	—	—	—	—	—	Butter 0·5	—
Time of cooking (mins.)	15	24	17	35	33	13	17
Highest temp.	100°	100°	100°	100°	100°	190°	175°

Steaming. In Exps. E and G, Kafuku's apparatus was used to measure moisture.

Baking. In Exp. F, the material (8 mm. thick) was kept at 190° for 3 minutes, and it appeared to be overbaked. In Exp. G, it was kept at 175° for 1 minute with good results.

[1] Contribution from Department of Domestic Economics, St Agnes' School for Girls (Heian Jogakuin), Kyoto, No. 2.

Gain and loss during cooking.

The sweet potato generally lost weight by cooking, especially in the case of baking, where the loss was due entirely to the removal of water.

The soluble sugar was lost in boiling or steaming, the loss in boiling being 0·4 % of the fresh material, or, about 5 % of the total soluble sugar.

Table II. *Loss by cooking.*

	Exp. No.	A	B	C	D	E	F	G
	Fresh wt.	105	98	97	98	97	63	76
	Dry wt.	—	90	95	100	99	101	101
Loss of sugars	Monosaccharides	0·6	—	—	0·2	—	—	—
	Disaccharides	0·6	—	—	0·2	—	—	—

The figures represent percentages of the respective fresh or dry weights before cooking.

Chemical changes during cooking.

It is known that starch, by the action of heat and water, is partly hydrolysed into soluble sugars. Such a partial hydrolysis of starch must be expected to occur in cooking sweet potatoes even if no water is added, as the tissue itself contains about 60 % moisture. Thus even in baking, one might expect increase in the amount of soluble sugars. Contrary to the expectation, the soluble sugars diminished considerably, and the starch-like substance increased instead. This fact is of great importance in discussing the nutritive value of the cooked food.

Table III. *Change of chemical composition by cooking.*

				Dry matter as 100				
Method of cooking	Exp. No.	Sample	Moisture %	Monosaccharides	Disaccharides	Dextrin	Starch	Cellulose
Boiling	A	Fresh	64	11·9	19·4	—	42·5	1·9
		Cooked	(67)	17·6	13·6	—	54·5	2·1
	B	Fresh	65	13·1	27·4	10·0	28·7	2·9
		Cooked	68	12·8	20·9	3·4	41·9	—
	C	Fresh	60	13·5	25·5	15·0	—	3·3
		Cooked	61	11·8	18·7	3·3	—	2·1
Steaming	D	Fresh	69	15·8	15·5	—	33·9	1·3
		Cooked	68	19·7	11·9	—	44·7	19·0
	E	Fresh	58	13·6	15·2	17·4	22·9	—
		Cooked	57	10·5	15·8	13·3	35·6	—
Baking	F	Fresh	67	12·7	20·0	—	11·9	15·0
		Cooked	47	12·5	18·1	—	11·7	1·0
	G	Fresh	62	13·4	17·1	24·5	28·9	—
		Cooked	49	12·2	14·1	17·6	38·8	—

Moisture. About 5 to 10 g. of the material were dried to a constant weight at 105°. The decrease in weight was assumed to be moisture.

Monosaccharides. About 20 g. of material was treated for 45 minutes with hot water. In the extract, reducing sugar was determined by Bertrand's method. Only in Exp. G, Willstätter's iodimetric method was adopted also.

Disaccharides. One part of the extract was hydrolysed with 3 % H_2SO_4 for 30 minutes on a water-bath. The increase in reducing power was assumed to represent disaccharides.

Dextrins. The rest of the extract was boiled with 3 % HCl for 2 hours

under reflux over a direct flame. The increase in reducing sugar less that derived from disaccharides was assumed to originate from dextrins.

Starch. The water-insoluble residue was hydrolysed by boiling with 3 % HCl for 2 hours. The reducing sugar thus formed was assumed to represent starch.

Cellulose. The residue from the starch was boiled for 30 minutes with 1 % KOH, filtered, and the solid, taken to be cellulose, was washed well with water, alcohol, and ether and dried to constant weight at 105°.

Cooking and digestibility.

The increase of insoluble polysaccharide and decrease of soluble sugars by cooking naturally suggest that cooked sweet potato would be more resistant towards the digestive enzymes. Contrary to expectation, the cooked sweet potato was more easily hydrolysable by diastase than the raw material. Further, by diastatic hydrolysis of the boiled material less dextrin and much more soluble sugar was formed.

Table IV. *Digestibility.*

A.

	Control*		Fresh material		Boiled material	
Time of digestion (hrs.)	0	2	0	2	0	2
Monosaccharides	21·7	20·3	20·5	37·5	34·3	98·8
Dextrins	49·9	55·3	51·8	78·5	56·9	68·6

B.

	Control*		Fresh material		Steamed material	
Time of digestion (hrs.)	0	2	0	2	0	2
Monosaccharides	21·9	32·1	22·6	35·6	33·3	84·2
Disaccharides	24·6	32·0	12·5	31·2	26·5	51·9
Dextrins	79·6	82·4	80·0	82·4	71·2	84·4

* Control for autolysis. Fresh material and boiled diastase solution were used.

Digestibility. The material was the same as was used in Exp. C, and that of the steamed one was the same as Exp. E. 400 cc. 1 % diastase (Pharmac. Jap.) was mixed with 20 g. of finely chopped material; in an aliquot of 150 cc. reducing sugar, disaccharides and dextrins were determined as described above. The remainder was kept for 2 hours in a thermostat at 40°, and the determination was repeated. The same was done with the steamed material. A control sample with boiled enzyme solution and fresh material was similarly treated. In measuring digestibility of steamed material, Willstätter's method was used to determine reducing sugars.

Discussion.

The peculiar increase in the starch fraction after cooking cannot be explained by loss of soluble sugars by diffusion because, even in Exp. B where the loss of dry weight is about 10 %, assuming that this 10 % loss is caused

by soluble sugars only, the apparent increase in starch cannot exceed 4 %, whilst the actual increase is, as is shown in Table III, more than 13 %.

Link and Tottingham [1923, 1925] observed a marked amount of caramelisation of sugars in plant tissues when they were heated to 80°. Since during cooking the temperature may rise as high as 230° in frying, considerable polymerisation of soluble sugars should take place, especially in the case of baking.

On the other hand, the easier digestibility means that the starch has undergone partial hydrolysis. The partially hydrolysed starch may remain in the starch fraction, if the degree of hydrolysis is slight. Thus, the apparent increase in the starch fraction and the easier digestibility after cooking can be explained by the polymerisation of soluble sugars and the partial hydrolysis of starch itself.

Daikuhara [1902] has reported that the taste of sweet potato is independent of the soluble sugar content of the raw material. It seems probable therefore that the increased sweetness of cooked sweet potato, important from a practical dietetic point of view, is connected with partial hydrolysis of the starch during cooking. Further experiments on this point are necessary.

Summary.

1. Chemical changes in the carbohydrates of sweet potato after boiling, steaming and baking were compared.

2. Sweet potato loses weight during cooking, especially by baking, owing to loss of moisture.

3. The decrease in soluble sugars and dextrins by cooking is most remarkable in baking.

4. The starch fraction always increases in cooking.

5. The digestibility of boiled and steamed material is much greater than that of the raw substance.

6. By the action of heat and water, the starch of sweet potato undergoes partial hydrolysis, whilst soluble sugars and dextrins polymerise to some extent.

The authors wish to express their sincere gratitude to Prof. Dr S. Komatsu of Kyoto Imperial University for his kind advice and valuable criticisms; to our colleagues Mrs S. Ueda and Miss U. Okamura, and pupils K. Inazumi, K. Yuhasi, K. Osikamo and N. Oka for their assistance during the experiment. Hearty thanks are due also to the Gunze Silk Filatory, Ayabe, for defraying the larger part of the cost of the experiment and to Miss H. Imamura, Sayama-mura near Kyoto, for supplying a part of the materials.

REFERENCES.

Link and Tottingham (1923). *J. Amer. Chem. Soc.* **45**, 439.
—— (1925). *J. Amer. Chem. Soc.* **47**, 470.
Daikuhara (1902). *Imper. Agric. Exp. Sta. Reports*, Tokyo, **22**, 139.

CCXIII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXII. THE ISOLATION AND CHARACTERISATION OF ERGOSTEROL FROM *PENICILLIUM PUBERULUM* BAINIER GROWN ON A SYNTHETIC MEDIUM WITH GLUCOSE AS SOLE SOURCE OF CARBON.

By JOHN HOWARD BIRKINSHAW, ROBERT KENNETH CALLOW and CATHERINE FÉLICITÉE FISCHMANN.

From the London School of Hygiene and Tropical Medicine, and the National Institute for Medical Research, London, N.W. 3.

(*Received October 24th, 1931.*)

Whilst sterols are generally known to occur in moulds, and the presence of ergosterol in a number of species grown on synthetic media has been demonstrated by the development of antirachitic activity on irradiation [Preuss *et al.*, 1931], ergosterol has not previously been actually characterised after isolation from a mould grown on a synthetic medium in which glucose is the only source of carbon.

In the case of the sterol isolated from *Penicillium puberulum* Bainier, it has been found that the physical properties of the sterol itself and the benzoate prepared from it correspond closely with those of purified ergosterol from yeast and ergosteryl benzoate, respectively, and final confirmation of its identity with ergosterol is afforded by measurements of the absorption spectrum and of the antirachitic activity acquired after irradiation. These observations are significant in yielding experimental evidence of the synthesis of ergosterol from glucose.

Experimental.

The organism employed was the strain of *P. puberulum* Bainier, used by Alsberg and Black [1913] in the preparation of penicillic acid, and was supplied by the courtesy of Dr Charles Thom, of the United States Department of Agriculture. Its history is given in Dr Thom's "Penicillia," p. 272.

The medium used was a Czapek-Dox solution containing glucose as sole source of carbon, and was identical in composition with the basal metabolism solution of Raistrick and Rintoul [1931]:

Glucose	50 g.
$NaNO_3$	2 g.
KH_2PO_4	1 g.
KCl	0·5 g.
$MgSO_4,7H_2O$	0·5 g.
$FeSO_4,7H_2O$	0·01 g.
Water	1000 cc.

The cultures were grown in 1000 cc. flasks, each containing 350 cc. of medium, and were incubated at 24°, or in trays each containing 5 litres in the incubator-steriliser designed for mould cultures by Birkinshaw, Charles, Lilly and Raistrick [1931]. In the latter case the temperature was maintained at 16–20°.

In order to obtain spores for sowing, Roux bottles containing Czapek-Dox agar were inoculated from the stock culture. When a good growth of spores was attained, 150 cc. of sterile water were added to each bottle, and the surface of the culture lightly rubbed with a sterile wire. The suspension was poured into a flask, and 2·5 cc. of this suspension were used for sowing each flask, or 100 cc. for each 5 litre tray. After an incubation period averaging 2–3 weeks, corresponding with the disappearance of about 90 % of the glucose present, the mycelium was separated from the metabolism solution by filtration, and after washing with water was squeezed out, and dried by exposing in a thin layer to the air at a temperature of about 25°. When most of the water had been removed in this manner, the mycelium was further dried *in vacuo* over $CaCl_2$ or H_2SO_4. When quite brittle it was finely ground in a mortar and extracted with ether in an apparatus of the Soxhlet type. A crystalline substance could be isolated from the extract showing an intense reaction for sterols. Further work was therefore undertaken with the object of identifying the sterol or sterols present.

In a preliminary examination made of the ether-soluble material from one batch of mould the residue left after evaporation of the ether was twice recrystallised from alcohol. The product (0·35 g.) was now not completely soluble in ether. The crystals obtained from the ethereal extract (0·28 g.) had M.P. 123–133°, $[\alpha]^{20}_{5461}$ − 122° in chloroform, gave an intense blue coloration with trichloroacetic acid characteristic of ergosterol [Rosenheim, 1929] and contained 73 % of sterol (calculated as ergosterol) precipitated by digitonin.

The ether-soluble material from another batch of mould was dissolved in a large volume of light petroleum, from which three fractions were obtained. (I) on cooling, white crystals (0·55 g.), (II) after evaporation (0·70 g.), and (III) a pasty residue after complete evaporation (2·25 g.). Fraction I did not redissolve completely in ether. The residue (I *a*) (0·1 g.) had M.P. 106–121° and contained nitrogen. The ethereal extract (I *b*) was evaporated to dryness. The following data were obtained for these fractions.

Fraction	M.P.	$[\alpha]^{20}_{5461}$ in $CHCl_3$	Free sterol precipitated by digitonin %	Reaction with CCl_3CO_2H
I *b*	127–139°	− 139·5°	70	Strongly +
II	65–110°	− 19·4°	7·2	Strongly +
III	Below 75°	− 6·3°	2·9	Weakly +

Fraction I *b* in 0·0025 % alcoholic solution gave an absorption spectrum similar to, but less intense than, that of ergosterol, with the following extinction coefficients: 9·9 at 290 *mμ*, 18·7 at 282 *mμ*, 18·7 at 271 *mμ*, 8·5 at 247 *mμ*. The corresponding figures for ergosterol are ϵ = 13·5, 25·6, 24·5, 6·6.

These data appear to indicate that the first fraction contains the bulk of the free ergosterol, whilst fraction II probably contains ergosterol largely as ester, and fraction III consists mostly of other compounds, possibly including other sterols.

0·25 g. of fraction I *b* was boiled with 10 cc. of 3 % alcoholic potassium hydroxide for a few minutes. After the addition of a little water crystals separated on cooling. These were collected and dried over calcium chloride; weight 0·134 g., M.P. 154–158°, $[\alpha]^{20}_{5461}$ − 162° in chloroform. Pure hydrated ergosterol has M.P. 160–163°, $[\alpha]^{20}_{5461}$ − 167·2° [Callow, 1931].

The benzoate was prepared by the addition of a slight excess of benzoyl chloride to 0·11 g. of the material dissolved in 3 cc. of pyridine. The product was isolated by pouring the reaction-mixture into water and recrystallised from ethyl acetate. It separated, in the manner characteristic of ergosteryl benzoate, in leaflets changing to bulky thin needles which had M.P. 161–163°, $[\alpha]^{20}_{5461}$ − 85·3° in chloroform. Pure ergosteryl benzoate has M.P. 169–171·5°, $[\alpha]^{20}_{5461}$ − 88·3°.

Finally, the benzoate was hydrolysed, the absorption spectrum of the resultant sterol was measured, and the antirachitic activity which it acquired on irradiation was determined. In 0·002 % alcoholic solution the extinction coefficients were 15·0 at 290 *mμ*, 28·4 at 282 *mμ*, 26·9 at 271 *mμ*, and 6·2 at 247 *mμ*. The curve showed the maxima characteristic of ergosterol, but with slightly greater intensity. A solution of the sterol in alcohol (0·01 %) stirred with a stream of nitrogen was irradiated in a silica cell by a mercury vapour lamp. A solution of pure ergosterol from yeast was irradiated under the same conditions for the same period and, after dilution in olive oil, the antirachitic potencies of the two products were compared on rats by the radiographic method described by Bourdillon *et al.* [1931]. Three pairs of rats were used, to which graduated doses were given, and three rats of the same litter were used as controls and given doses of a standard solution of vitamin D. The antirachitic potencies of the two products were found to be equal.

An estimate of the yield of ergosterol from the mycelium was made in a typical experiment, using 100 flasks (*i.e.* 35 litres of medium) and incubating at 24° for 20 days. The dry mycelium weighed 391 g. and yielded an ether extract weighing 19·9 g. after removal of solvent *in vacuo*. The solid portion of the extract was collected and dried on a porous plate. It weighed 1·8 g., and in alcoholic solution gave the absorption spectrum characteristic of ergosterol. The intensity of absorption indicated the presence of 28 % of ergosterol, corresponding to 0·13 % in the dried mycelium.

Our thanks are due to Mr J. St L. Philpot for measuring the absorption spectra, which were obtained by the method described by Bourdillon *et al.* [1930].

We are also grateful to Prof. H. Raistrick and Dr O. Rosenheim for their interest in this work.

REFERENCES.

Alsberg and Black (1913). *U.S. Dept. Agric. Bur. Plant Industry Bull.* **270**, 1.
Birkinshaw, Charles, Lilly and Raistrick (1931). *Phil. Trans. Roy. Soc. Lond.* B **220**, 127.
Bourdillon, Bruce, Fischmann and Webster (1931). *Medical Research Council Special Report No.* **158**.
—— Gaddum and Jenkins (1930). *Proc. Roy. Soc. Lond.* B **106**, 388.
Callow (1931). *Biochem. J.* **25**, 79.
Preuss, Peterson, Steenbock and Fred (1931). *J. Biol. Chem.* **90**, 369.
Raistrick and Rintoul (1931). *Phil. Trans. Roy. Soc. Lond.* B **220**, 2.
Rosenheim (1929). *Biochem. J.* **23**, 47.

CCXIV. DETERMINATION OF SMALL AMOUNTS OF PHOSPHATIDES AND CHOLESTEROL IN TISSUES[1].

By MAURICE JOWETT and ERIC WILLIAM LAWSON.

From the Muspratt Laboratory of Physical and Electro-Chemistry, University of Liverpool.

(*Received October 24th, 1931.*)

Although there have been many publications on methods of estimating phosphatides and cholesterol in tissue, the value of many of the methods described remains uncertain. In some cases extraction of lipoid substances may be incomplete. Not all of the phosphorus in extracts may be derived from phosphatides. Colorimetric methods of doubtful validity [Nekludov and Chalatov, 1929] are still in use for determining cholesterol. While micro-gravimetric methods have been worked out for pure cholesterol, they have received little application to tissue-extracts.

Before undertaking analyses of tissues we have thought it advisable to make an investigation of methods of analysis. This is reported here with necessary details, together with such results of the analyses as are of methodical interest.

An account of analytical methods is necessary to make it possible to assess the value of the analytical data given in an accompanying paper [Jowett, 1931]. Some results of interest in themselves have also been obtained.

Extraction of lipoid substances from tissues.

In work carried out some years ago in which one of the authors took part, dried tissues were extracted with chloroform[2]. While such a solvent has the advantage that it is likely to extract little phosphorus beyond that contained in phosphatides, an impression prevails that chloroform and ether may not give complete extraction of lipoid substances.

We have therefore tested this point. Fresh rabbit liver was minced, rubbed in acetone, and dried as described later. After exhaustive extraction

[1] This investigation was undertaken on behalf of the Liverpool Medical Research Organisation (Director: Prof. W. Blair Bell).

[2] This method was adopted from Bullock and Cramer [1913–14], who in turn appear to have followed Smith and Mair [1911, 1913]. Dr Cramer kindly sent us a description of the methods used. The results obtained by us have been summarised in several places, *e.g.* Bell [1925, 1930] and Lewis [1927]. They are not further detailed in the accompanying paper [Jowett, 1931], since they are less accurate than the data given there.

in a Soxhlet apparatus with chloroform, the tissue was extracted with absolute alcohol[1]. The alcohol extract was taken to dryness and re-extracted with chloroform. If alcohol is assumed to complete the extraction of lipoid substances, the results show that chloroform extracts only about 82 % of the lipoids from rabbit liver.

With other tissues the proportion of the lipoids extracted by chloroform may be as low as 50 %. This is suggested by a comparison of two series of estimations on human uterine muscle, which has a fairly constant composition. In the first series, made some years ago with chloroform, the average total lipoid content found for five specimens was 3·5 %. In the present work with absolute alcohol as solvent, five specimens gave an average value of 7·2 % for total lipoids.

In the present work we have used absolute alcohol to extract lipoid substances from tissues and as the result of some preliminary experiments decided to extract the tissues without drying them thoroughly.

The tissue for analysis, weighing from 5 to 20 g., is cut up, and gross fat, connective tissue and so forth removed as far as possible. It is then finely minced in a Latapie type mincer of small size. The minced tissue is weighed after removing a portion for dry weight determination, and then covered with 20 to 80 cc. of acetone or absolute alcohol, the tissue being well stirred up immediately to avoid caking. When convenient, the material is poured into a Soxhlet thimble of double thickness, and washed with alcohol. The filtrate is retained. The thimble is mounted with wire in a conical flask under a reflux condenser, and its contents are extracted three times, for an hour each time, with boiling absolute alcohol. Fresh alcohol is used for each extraction.

The combined extracts are taken to dryness, alcohol being first distilled off, and the operation then being finished at as low a temperature as possible in a stream of carbon dioxide or nitrogen. If the temperature is too high the lipoid material obtained will not dissolve completely in chloroform; if removal of alcohol is incomplete, the chloroform extract may be turbid. The dried alcohol extract is extracted by boiling with three successive portions of chloroform for a few minutes, and the filtered chloroform extract kept for analysis.

The alcohol extraction is fairly complete. In two cases the tissue after alcohol extraction was dried, ground up and extracted with ether for 3 hours. The weight of the ether extract amounted to an additional 0·5 % for some malignant tissue, and 2·4 % for a sample of muscle.

Estimation of dry weight and total lipoid content of tissues.

Estimation of dry weight. The minced tissue is stirred with a glass rod to a uniform paste, and a portion of about 0·5–1·0 g. removed to a small, light

[1] All solvents used in this work were of good quality; before use they were dried with calcium chloride (quicklime in the case of alcohol), and redistilled using a twelve-pear column.

weighing-bottle. After being weighed, the tissue is covered with acetone, and the acetone is afterwards roughly evaporated off in a stream of nitrogen or carbon dioxide. The bottle is then dried to constant weight in the vacuum oven over sulphuric acid at 40° ; this requires about 5 days. Bottles were weighed on a micro-balance to 0·01 mg., and the change in weight of a bottle to which pure acetone was added was applied as a correction. The weights of empty bottles were reproducible within 0·2 mg., and the weights as corrected are probably more accurate than this. This would suggest that dry weight estimations are accurate within about 0·2 %, but difficulties of sampling and of reproducing the degree of dryness may make the accuracy less than that figure.

Estimation of total lipoids. The chloroform extract from a specimen of tissue is brought to a volume of 100 cc. immediately before an estimation. This is necessary, as chloroform is very expansible and volatile.

A measured volume of the extract is transferred by pipette to a weighing-bottle, which is then immersed in a water-bath at 50–55°, and a stream of carbon dioxide led over the surface of the liquid until at least 10 minutes have elapsed after evaporation of chloroform appears to be complete. After being wiped, the bottle is transferred to a desiccator containing calcium chloride and weighed soon afterwards. Every time an estimation was made, a bottle was put through the same process with pure chloroform, and its change of weight was applied as a correction to the other changes; no regular change of weight took place, and the change was usually less than 0·3 mg. The weight of lipoids taken for an estimation was from 20 to 100 mg. Duplicate estimations, which were always made, checked very closely.

Fresh estimations of the total lipoid content of the chloroform solutions were always made when further volumes of the solutions were taken for analysis[1].

The estimation of phosphatides.

It is uncertain that phosphatides are the only phosphorus-containing substances extracted from tissues by absolute alcohol, and we are relying on phosphorus estimations to determine phosphatides. We therefore undertook experiments with the object of separating phosphatides on the small scale from other phosphorus-containing substances.

According to Nerking [1910] acetone does not precipitate lecithin completely. Nor, according to experiments made by us, does cadmium chloride in alcoholic solution completely precipitate small amounts of lecithin.

Nerking states, however, that magnesium chloride can precipitate lecithin completely in an acetone-ether mixture, and we commenced experiments to test this. The work of Bloor [1929] came to our notice soon afterwards, and

[1] Methods of handling small quantities of liquids are described by Szent-Györgyi and Tominaga [1923], and we have found these helpful in designing our methods.

most of our work took the form of testing the modification of Nerking's method described by this author.

Estimation of phosphorus. Our estimations of phosphorus have been made by the colorimetric method of Briggs [1924].

The method was modified by keeping the quinol and the sodium sulphite in the same solution, 0·5 % quinol in 10 % sodium sulphite. 2 cc. of the solution are added for a total volume of 10 cc.

Extensive testing with inorganic phosphate solutions showed us that an accuracy of about 1 % can usually be obtained, when the amount of phosphorus is within some 30 % of that in the standard, which is best taken at 0·06 mg. P (as potassium dihydrogen phosphate). The Klett top-reading colorimeter, provided with a lamp, was used. Preliminary estimations are necessary when the amount of phosphorus to be estimated is quite unknown.

Estimations of organic phosphorus were made after ignition with sulphuric acid and hydrogen peroxide. The chloroform solution is taken to dryness in a 6-inch thick-walled test-tube in a stream of air, 0·3 cc. of concentrated sulphuric acid is added, followed by 0·5 cc. of Merck's perhydrol previously redistilled under low pressure. The ignition is carried out over a small flame, more peroxide being added as necessary, and the tube being rotated to rinse the walls towards the end of the ignition. After addition of water, the acid is neutralised approximately with sodium hydroxide.

Tests on the ignition of known amounts of inorganic phosphate gave complete recovery. With lipoid phosphorus the reproducibility was often within 1 %, and usually within 3 %.

Experiments with pure lecithin and magnesium chloride. We shall report experiments only in which the precipitation took place under the conditions used by Bloor, as the variations tried did not greatly affect the results.

From 1 to 3 cc. of an alcoholic solution of lecithin[1] was taken to dryness in a centrifuge-tube, and dissolved in 2 cc. of light petroleum and 7 cc. of acetone. Then was added 0·1 cc. of a saturated solution of hydrated magnesium chloride in absolute alcohol. After thorough stirring of the precipitate—precipitation of the salt takes place independently of the presence of lecithin—it was centrifuged down, the supernatant liquid poured off, and the precipitate washed with 2 cc. of acetone.

Phosphorus was estimated in the supernatant liquid and wash liquid; it was usually necessary to unite the liquids from several precipitations and to add some inorganic phosphate after ignition, in order to obtain enough phosphorus to estimate.

A "blank" correction was necessary; when no lecithin was used in the precipitation and washing, phosphorus was apparently present in the supernatant and wash liquids.

[1] The lecithin was made according to the method of Levene and Rolf [1927] by Dr H. I. Price, to whom we are indebted for it.

The figures obtained are given in Table I.

Table I. *Precipitation of lecithin by magnesium chloride.*

Mg. P taken	Mg. P not precipitated	Mg. P (blank)	Corrected mg. P not precipitated	% of P not precipitated
0·111	0·0086	0·0022	0·0064	6
0·333	0·0231	,,	0·0209	6
0·114	0·0099	,,	0·0077	7
0·114	0·0096	,,	0·0074	7
0·342	0·0435	,,	0·0413	12
0·113	0·0080	,,	0·0058	5
0·113	0·0108	,,	0·0086	7
0·067*	0·0045	0·0026	0·0019	3
0·067*	0·0047	,,	0·0021	3
0·134*	0·0085	,,	0·0059	4
0·134*	0·0058	,,	0·0032	2
0·067*	0·0037	0·0024	0·0013	2
0·067*	0·0039	,,	0·0015	2

* For these estimations 200 mg. of lecithin were precipitated with $MgCl_2$ and washed as in the micro-estimations. The precipitate was extracted with ether and chloroform, and the extract finally dissolved in chloroform together with a little alcohol.

Evidently about 93 % of the lecithin is precipitated, or about 97 % when the lecithin has been purified.

Experiments were then made to test whether the reason for incomplete precipitation was solubility of the lecithin in the precipitant mixture. Magnesium chloride precipitates, after the usual washing, were washed again with a mixture of 7 cc. of acetone and 2 cc. of light petroleum, and phosphorus estimated in this liquid. When 0·113 mg. of lecithin-P was taken initially, and two washings made, the uncorrected P from two washings was about 0·0061 mg. as a mean. Subsequent pairs of washings gave about the same value. The value suggests that most of the lecithin that remains unprecipitated does so on account of its solubility.

According to Bloor the lecithin may be extracted from the magnesium chloride precipitate with moist ether, solution being, however, a slow process. The water in the ether dissolves the magnesium chloride to form a separate phase.

We have found it very difficult to extract all the lecithin with ether. It does not dissolve completely, but can be decanted suspended in ether. From three to five washings with ether are necessary. The recoveries of phosphorus were then 88, 96, 91 and 89 % of that taken. These values are moderately satisfactory, but the procedure is too laborious to be a practical method.

Extraction with chloroform was then tried: the solvent was brought to boiling-point and extraction made three or four times. This is a simpler method, but the recovery is variable. The percentage recoveries found were 88, 86, 85, 96, 94, 89, 88, 83, 75, 92 and 78.

We have also tried estimating the phosphorus in the magnesium chloride precipitate by igniting this directly. The recovery is usually from 95 to 102 %, but the ignition in presence of magnesium chloride is lengthy and laborious.

Our conclusion is that the most satisfactory way of using the magnesium chloride method is to estimate total phosphorus and subtract from it phos-

phorus not precipitated by magnesium chloride. This variant has been used in the work of the following section.

Experiments with lipoid mixtures and magnesium chloride.

The magnesium chloride precipitation has been made on tissue extracts, containing phosphatides, cholesterol and fats.

The data obtained are given in Table II. "Blank" corrections were not applied, and if the behaviour were the same as it is for pure lecithin about 5 % of the phosphorus would have remained unprecipitated.

Table II shows that in some cases the percentage not precipitated is much higher. It is highest when the fat content of the extract is high (shown by a high value of the ratio of total lipoids to total phosphorus).

Table II. *Precipitation in lipoid mixtures.*

Tissue extract	Total lipoids / Total P	% P not precipitated
2 A	125	26·0
2 B	317	20·0
2 C	343	18·5
6 B	175	15·2
5 B	43	11·3
5 A	50	9·3
7 B	41	8·6
3 A	48	5·9
5 C	33	5·6

Now there is no special reason to believe that, in the presence of fat, alcohol will extract from tissues non-phosphatide-phosphorus that would otherwise remain unextracted.

The most probable conclusion is that fat interferes with the precipitation of phosphatides by magnesium chloride.

If this is so, Bloor's method for estimating phosphatides will tend to give low results, quite apart from the fact that the ether extraction in our hands has not given complete recovery.

Conclusion. We have been forced to assume that the whole of the phosphorus in our extracts represents phosphatides. The assumption is probably not much in error, if we may judge from a comparison between the results of the magnesium chloride method on pure lecithin and on tissue extracts containing little fat. This comparison confirms the general impression that anhydrous solvents extract little phosphorus-containing material other than phosphatides.

We have calculated the phosphatide content of our extracts on the basis that the phosphorus content of phosphatides is 4·00 %. This figure may be a little too high—Levene and Rolf [1927] find about 3·9 % for pure lecithin, and Levene and West [1916] about 3·7 % for kephalin—but the error introduced should tend to offset any error in assuming that all the phosphorus found represents phosphatide.

The phosphorus percentage will vary somewhat in phosphatides from

different tissues, but the available data suggest that the variation is not great. The objections that have been raised to assuming a constant composition seem to have been over-emphasised.

The estimation of cholesterol.

The gravimetric method, in which cholesterol is combined with digitonin, has been used.

The compound has been precipitated in a manner similar to that employed by Szent-Györgyi [1923]. A quantity of material containing from 0·5 to 3 mg. of cholesterol is taken to dryness in a 10 cc. centrifuge-tube, dissolved in 2 cc. of acetone, and 1 cc. of digitonin solution added (1 g. of commercial digitonin dissolved in 50 cc. of 80 % alcohol). After being stirred and warmed, the liquid is evaporated to about one-half in a stream of air. After about 15 minutes the precipitate is washed in the centrifuge-tube, in a manner not very different from that of Tominaga [1925], twice with 7 cc. of ether, and the supernatant liquid poured off into the filter-tube. Three washings are then given in the heat with 7 cc. (each time) of a mixture containing 80 % of acetone and 20 % of water. Of the wash-liquids it is only the 80 % acetone that removes much of the excess digitonin.

The precipitate is then washed on to the filter with about 10 cc. of pure acetone, followed by 6 cc. of ether. The filter-tube is then left in an electrically heated oven at 105–110° for an hour. On removal it is capped and left in a calcium chloride desiccator for 20 minutes, after which it is weighed.

Experiments were first made with Jena sintered glass filter-tubes. Unfortunately the filters with the finest pores (G 4) often became clogged with the precipitate, while those with larger pores (G 3) allowed the precipitate to pass through to some extent. The use of sintered glass filters was therefore abandoned.

The type of filter-tube described by Szent-Györgyi [1923] was employed, but lighter caps were used which were flat at the top and had somewhat flattened sides. The filter-tubes weighed, complete, about 5 to 6 g. The asbestos used was Powminco Grade xx; a little solid asbestos was followed by an aqueous suspension, and stronger suction succeeded gentle suction. After washing with water, acetone and ether the tubes were heated and then weighed; this procedure was repeated until the weight became approximately constant. The weight usually becomes constant within 0·04 mg., but further washing would result in further loss of weight.

Similarly when cholesterol digitonide is on the filter, continued washing leads to a larger and continued loss of weight. Any method of using the filter-tubes has to be established empirically.

To test the method described above a series of estimations was made on pure cholesterol[1]. A solution in absolute alcohol containing 1·021 mg. per cc.

[1] We are indebted for the cholesterol to Mr W. Doran, Department of Organic Chemistry, University of Liverpool.

was used to obtain the data in Table III. The average deviation of the estimations from the "theoretical" value (taking the precipitate to contain 25 % of cholesterol) is about 3 %. Tominaga claimed an accuracy within 1 %. Probably we could have obtained greater accuracy if every filter had been prepared with extreme care, many being rejected, and if those accepted had been used for only a few estimations. We have used a filter about eight times before renewing the packing.

Table III. *Weight of digitonide (mg.) from cholesterol.*

	1 cc.	2 cc.	3 cc.
	4·04	8·50	11·95
	3·91	8·66	12·58
	3·96	8·12	—
	4·11	7·94	—
	3·87	—	—
Mean	3·98	8·30	12·26
Theoretical	4·08	8·17	12·25
Average percentage deviation from theoretical	2·8	3·4	2·6

Estimations on tissue extracts. Although several papers have been published on estimation of small amounts of cholesterol by the gravimetric method, and the methods were apparently designed for estimating cholesterol in tissue extracts, the method seems to have been little applied to that purpose.

Our experience of the application of the method has not been very satisfactory.

Free cholesterol. When much fat in relation to cholesterol is present, the precipitate is contaminated with fat. We have not judged it wise to remove, prior to the estimation, the fat which often precipitates on cooling a hot acetone solution, as this may contain cholesterol. The fat is removed by washing the precipitate as described, removal being most rapid in the heat.

An examination of the results of a series in which two estimations were made on each of a number of tissue extracts showed that the presence of much fat is associated with less accurate agreement between the results. When the ratio of total lipoids to cholesterol was over 20 (averaging about 50), the average difference between the two values was 8·7 %. With lower values for the ratio (about 8–12), the average difference between the two values was only 4·3 %. Evidently the presence of much fat leads to irregular values.

When several estimations on the same tissue extract have been made with different quantities, there is a certain tendency for lower relative values to be found, the more the material taken for an estimation. This appears to indicate that fat tends to inhibit formation of a digitonide precipitate that is retained by the filter.

Total cholesterol. A portion of the tissue extract is taken to dryness, and then there is added to it 1 cc. of ether followed by 1 cc. of 15 % sodium ethoxide in absolute alcohol. After being warmed to dissolve the lipoid material, the solution is allowed to stand at least 24 hours. According to

Gardner and Fox [1924], hydrolysis of cholesterol esters should then be complete. The solution is taken down to dryness, and 2 cc. of 2*N* sulphuric acid are added. After shaking, the material is extracted four times with ether in the test-tube used. The ether extract is evaporated to dryness.

Estimation of cholesterol is then carried out in the same way as for free cholesterol, except that the extract has been dissolved in 2 cc. of ether instead of acetone. Ether was used instead of acetone with a view to lessening the interference of fatty material with the precipitation, as most of the fat then stays in solution. The precipitate of digitonide is more flocculent with ether.

Preliminary estimations, made to test whether the use of ether gives the same results as acetone on free cholesterol, are given in Table IV. The data also serve to show the kind of reproducibility the methods give. The acetone and ether methods give very similar results, as far as the data go. Further tests would be worth while.

Table IV. *Comparison of acetone and ether methods on free cholesterol.*

	mg. precipitate acetone method	mg. precipitate ether method
Pure cholesterol	12·79	12·56 12·67
Tissue extract containing much fat	6·87 6·64	7·03 7·09
Tissue extract containing little fat	8·16 8·01	7·50 7·82
" "	8·52 8·84	8·78
" "	12·94 13·47	13·42 12·90

An examination of results, similar to that made in the case of free cholesterol determinations, showed that the extracts containing much fat did not tend to give more irregular results than the others.

The series shows an average difference of 5·4 % between the results of the two estimations on each extract; for the free cholesterol series the corresponding figure was 5·8 %. When the estimations on pure cholesterol in Table III are taken in pairs the average difference between the results for the two members of each pair is 4·0 %. The mean result of each pair of estimations diverges on the average from the theoretical value by 1·8 %, or by half the previous figure.

The values obtained from the pairs of estimations on free and bound cholesterol in tissues should therefore be on the average less than 3 % from the true value, apart from the existence of a systematic error, such as a tendency of fat to produce low values. It must be pointed out, however, that further repetitions of some of the estimations sometimes show much larger discrepancies.

Comparison with other work. Osato and Heki [1930] have made microgravimetric estimations of cholesterol in tissues, but do not yet appear to have published details of their method. The only other work on this subject

we are aware of is that of Onizawa [1928], who extracted cholesterol from tissues by a modification of Fox's method, thereby removing fats. His results on pure cholesterol and on tissues are apparently more reproducible than ours. The only apparent difference in the estimation of cholesterol is that this author washed his precipitates finally with water and dried them in a vacuum oven, as did Szent-Györgyi. Our results suggest that removal of fat prior to the estimation should lead to greater reproducibility.

SUMMARY.

Methods are described for the extraction and estimation of phosphatides and cholesterol in 5–20 g. of tissue.

Chloroform does not extract lipoid substances completely from dried tissues. Magnesium chloride precipitates pure lecithin almost completely, but the presence of much fat appears to interfere with precipitation of phosphatides. The analytical method described by Bloor [1929] probably gives low results. The phosphorus in our extracts is probably almost entirely derived from phosphatides.

The micro-gravimetric method has been tested on pure cholesterol and applied to tissue extracts. Much fat lessens the accuracy of free cholesterol determinations, but in the presence of ether does not appear to interfere with estimations of total cholesterol.

In conclusion the authors wish to acknowledge their indebtedness to Prof. W. C. M. Lewis for his interest in this work, and to Prof. H. J. Channon for some helpful advice.

REFERENCES.

Bell (1925). *Lancet*, ii, 1003.
—— (1930). Some aspects of the cancer problem. (London.)
Bloor (1929). *J. Biol. Chem.* **82**, 273.
Briggs (1924). *J. Biol. Chem.* **59**, 255.
Bullock and Cramer (1913–14). *Proc. Roy. Soc. Lond.* B **87**, 236.
Gardner and Fox (1924). *Biochem. J.* **18**, 1058.
Jowett (1931). *Biochem. J.* **25**, 1991.
Levene and Rolf (1927). *J. Biol. Chem.* **72**, 587.
—— and West (1916). *J. Biol. Chem.* **24**, 41, 111.
Lewis (1927). *J. Cancer Research*, **11**, 16.
Nekludov and Chalatov (1929). *Biochem. Z.* **208**, 60.
Nerking (1910). *Biochem. Z.* **23**, 262.
Onizawa (1928). *J. Biochem.* (*Japan*), **10**, 45.
Osato and Heki (1930). *J. Biol. Chem.* **87**, 541.
Smith and Mair (1911). *J. Path. Bact.* **16**, 130.
—— —— (1913). *J. Path. Bact.* **17**, 609.
Szent-Györgyi (1923). *Biochem. Z.* **136**, 102, 107.
—— and Tominaga (1923). *Biochem. Z.* **146**, 226.
Tominaga (1925). *Biochem. Z.* **155**, 119.

CCXV. THE PHOSPHATIDE AND CHOLESTEROL CONTENTS OF NORMAL AND MALIGNANT HUMAN TISSUES[1].

By MAURICE JOWETT.

From the Muspratt Laboratory of Physical and Electro-Chemistry, University of Liverpool.

(*Received October 24th, 1931.*)

Phosphatides and cholesterol are invariably constituents of animal tissues. They play a part in fat metabolism [MacLean and MacLean, 1927], and may be to some extent storage materials, but the work chiefly of Mayer and Terroine has shown that this is true only to a limited extent, and that they tend to maintain constant their quantities in any one tissue.

It is natural, then, that a rôle in regulating protoplasmic structure and function has been attributed to them. Of the modes of approach only one will be discussed here, which is derived from the facts that lecithin and cholesterol lower the interfacial tension between oil and water [Corran and Lewis, 1924; Okunev, 1928], and are emulsifying agents. Lecithin favours a dispersion of oil in water and cholesterol a dispersion of water in oil, the two being in this sense antagonistic, as shown by Corran and Lewis.

Similar action may well take place in the cytoplasm, particularly in cell-membranes. Corran and Lewis have expressed the view that a cell of which the contents show a high lecithin-cholesterol ratio is likely to be more permeable to water-soluble substances than a cell with a low ratio. This view receives some support from the fact that lecithin (or substances derived from it) favours haemolysis and cholesterol inhibits it *in vitro*. Too much lecithin presumably makes the membrane too labile for continued existence, while cholesterol counteracts the effect. Such effects may also be the reason for the retarding action of lecithin and the accelerating action of cholesterol on the growth of animal tumours found by Robertson and Burnett [1913], Moravek [1927] and Rondoni [1930].

The lipin content of a cell may, on the view of Corran and Lewis, influence its supply of building materials and metabolites.

This may permanently affect its general activities. In this way variation in lipin content might be one of many causal factors in the varied cell-differentiation that takes place in embryonic development, and might remain

[1] This investigation was undertaken on behalf of the Liverpool Medical Research Organisation (Director: Prof. W. Blair Bell, University of Liverpool).

in the adult cell as an expression of the variation in development. Cells having in some respects higher metabolism or more complex structure would be expected to have the higher lipin ratio.

An alternative view can be suggested on functional grounds. In the resting cell permeability to water-soluble substances may not be a limiting factor in maintenance of structure and metabolism. The growing cell, or more active resting cell, may require greater permeability to obtain its supplies. Hence the growing cell, or resting cell of high metabolism, may show a high lecithin-cholesterol ratio.

Yet little evidence can be offered that insufficient permeability hinders the mammalian cell from obtaining supplies.

The possible consequences of the antagonistic emulsifying properties can be developed in another way, not involving permeability. Although lipins probably tend to concentrate in cell-membranes, most of the material will usually remain in the interior of the cell, and may there share in regulating the state of aggregation of protoplasm. Lecithin is likely to favour fine dispersion in their aqueous medium of the protein-lipin colloids of protoplasm, and cholesterol is likely to tend to aggregate them. Finer dispersion will probably be accompanied by greater hydration. It may thus be argued that a high lecithin-cholesterol ratio will be found in cells of high water-content, high lability and high enzymic activity—since fine dispersion gives more available internal surface. These characteristics in a general way are those of young and growing cells, which have a high water-content and a high metabolic activity, and which must possess a labile structure to be able to undergo the rearrangements that precede cell-division.

Some evidence can be put forward in favour of the rôles attributed above to lipins. From a general colloid-chemical point of view their peptising effects are not likely to be restricted to oil-water systems, and work such as that of Handovsky, Lohmann and Bosse [1925] and of Theorell [1930] shows that there is very close association between lipins and serum or plasma proteins; Theorell finds that the less easily salted out the protein is, the higher is the phosphatide-cholesterol ratio of the associated lipin. Likewise, *in vitro* they affect the activity of enzyme extracts[1].

In the present paper are presented analyses of human tissues which, as far as they go, and in conjunction with already published data, tend to confirm the view that growing cells, in particular malignant cells, have a high phosphatide-cholesterol ratio. This is a finding not restricted to such cells; and there are other findings that theory does not predict. No exact and simple confirmation can be expected: factors other than lipins are concerned in

[1] Tsuneyoshi [1927] finds that lecithin increases and cholesterol diminishes (1) the consumption of oxygen by tissue powder in presence of succinic acid, and (2) the oxidation of glycine on charcoal. Eichholtz [1924] finds that lecithin promotes catalase action in serum. Cholesterol retards peptic action [Dörle, 1923]. However lecithin sometimes retards enzymic activity, as in the case of tryptic digestion [Hagihara, 1924; Standenath, 1925].

growth and metabolism. In addition, the material analysed seldom contains only one type of cell; our methods do not distinguish lecithin from other phosphatides, which may have quite different surface effects; nor is the capillary effect of cholesterol esters clear.

Experimental.

The tissues examined were removed at operations in Liverpool. The author is indebted for them principally to Prof. W. Blair Bell, Mr M. M. Datnow and Miss G. Griffith. Histological examinations of representative pieces of each tissue were made by Mr M. M. Datnow.

To avoid chemical change, tissues were minced and immersed in alcohol or acetone as soon as possible; this was complete in from 1 to 3 hours after excision for all but five specimens, which were kept overnight in a refrigerator.

The analytical methods have been described in an accompanying paper [Jowett and Lawson, 1931]. Mr E. W. Lawson assisted in the analytical work. Estimations were carried out in duplicate (with the exception of estimation of dry weight), and frequently several times when agreement was not good.

Tissues were sometimes kept some time in alcohol, and extracts were usually kept in chloroform for a considerable time, before the analyses were complete. Serious changes are unlikely to occur under these conditions.

Discussion of analytical results.

The analytical data that have been obtained are given in Table I. The tissues are seldom composed almost entirely of one type of cell. Some of the malignant tissues are diluted with fibrous tissue; one specimen of malignant tissue and some of the muscular tissues are diluted with fat[1]. Comparisons are made more difficult, but a number of conclusions can nevertheless be drawn.

According to many workers, for instance Cramer [1916], the water-content of young and of malignant tissues is higher than that of resting tissues, but in the present data this tendency seems usually to be obscured for malignant tissue.

In the present work, malignant tissues and normal epithelial tissues show on the whole higher contents of phosphatides and of cholesterol than do fibromyomata and normal muscle.

Of the seven specimens of malignant tissue, those containing the highest proportion of malignant cells have definitely the highest content of phosphatides and cholesterol, and tend to show the highest ratio of phosphatides to free cholesterol.

The data for cholesterol show a number of discrepancies, free cholesterol being sometimes found higher than total cholesterol, but nevertheless the conclusion may be drawn fairly definitely that of the tissues analysed malignant

[1] In Table I (last column), fat has been calculated by subtracting from the total lipoids the phosphatides and cholesterol; the figure will include fatty acids bound to cholesterol.

tissues are the only class in which the proportion of cholesterol in the ester form is considerable, averaging nearly 40 %.

Chorionic villi, according to Bell [1930], share many of the properties of malignant tissue. Of the two specimens analysed, the one obtained at an early period of pregnancy and containing a large proportion of syncytium should show these characteristics more than that at a later period, which

Table I.

No.	Tissue	% dry weight	% on dry weight of: Total lipoids	Phosphatides	Cholesterol: Free	Cholesterol: Total	Phosphatides: Free cholesterol	% cholesterol as esters	% fat in total lipoids
	Malignant:								
11 A	Carc. kidney (all malignant)	22·3	29·89	11·70	1·238	2·026	9·45	39	54
15 A	Sarc. uterus (all malignant)	17·5	19·51	8·98	1·667	2·64	5·4	37	40
3 A	Carc. omentum (⅔ growth)	18·65	17·42	9·12	1·545	2·23	5·9	31	35
2 A	Carc. breast (40 % growth)	20·7	27·95	5·58	0·827	1·389	6·75	40	75
8	Carc. caecum (half fibrous tissue)	15·15	13·46	5·51	1·113	1·759	4·95	36	46
9 A	Carc. breast (half fibrous tissue)	20·1	14·71	5·36	1·138	2·096	4·7	46	50
14 A	Carcinoma (growth ¼, blood clot ¾)	12·5	9·22	4·06	1·053	1·208	3·9	13	43
	Fibromyomata uteri:								
5 B	All healthy growth	21·0	4·66	2·71	0·544	0·619	5·0	12	29
10 A	All healthy growth	24·5	3·38	1·395	0·320	0·286	4·4	?	50
7 A	Hyaline degeneration of half growth	19·5	2·84	1·45	0·247	0·344	5·9	28	37
16 B	Healthy growth, but chiefly muscle	20·5	7·48	5·93	0·753	0·605	7·9	?	13
	Epithelial tissues:								
5 C	Normal ovary	19·0	11·04	8·25	0·727	0·835	11·35	13	17
11 B	Normal kidney	20·2	17·58	8·04	1·522	1·587	5·3	4	45
17	Chorionic villi, 10–12 weeks	11·94	16·33	8·24	1·407	1·492	5·8	6	40
13	Chorionic villi, 7 months	15·5	10·90	5·10	1·133	1·101	4·5	0	43
	Uterine muscle (healthy):								
5 A	—	21·2	6·55	3·30	0·749	0·851	4·4	12	37
7 B	With a little mucosa	19·3	4·97	3·04	0·606	0·636	5·0	5	36
10 B	—	19·7	7·38	2·97	0·818	0·595	3·6	?	51
15 B	With a little mucosa	21·4	8·65	3·77	0·788	—	4·8	—	45
16 A	—	18·2	8·38	5·42	0·936	0·935	5·8	0	24
	Muscle:								
2 B	Pectoral	25·9	33·74	2·66	0·537	0·717	4·95	25	90
2 C	Pectoral	24·9	29·24	2·13	0·615	0·901	3·5	33	90
9 B	Striated, pectoral	27·1	25·19	2·84	0·291	0·242	9·8	?	88
14 B	Leg	15·8	11·03	5·74	1·042	0·843	5·5	?	40
14 C	Leg	22·5	7·38	2·83	0·264	0·235	10·7	?	59
6 B	Bowel muscle, ¼ mucosa	17·1	23·85	3·40	0·660	0·628	5·15	?	83

contains little syncytium. Actually the younger has a greater water-content, a higher lipin content and a higher phosphatide-cholesterol ratio. Neither specimen contains an appreciable amount of cholesterol esters[1].

Associated with growing tissues we therefore appear to have a high lipin content and a high phosphatide-cholesterol ratio, but these are not restricted to such tissues. In the present data normal ovary[2] and normal kidney have

[1] Bienenfeld [1912] found a large proportion of bound cholesterol in early placentae, but her extraction appears to have been incomplete.

[2] The tissue analysed contained approximately: lutein tissue 2 parts, corpora albicantes 4, and ovarian stroma 11.

a high lipin content; ovary and two specimens of muscle have a very high phosphatide-cholesterol ratio.

That these quantities have nevertheless a definite significance for growing tissue of an abnormal nature can be shown in another way, by comparing abnormal tissue with the neighbouring tissue from which it may have arisen. Theoretically, the comparison should probably be made between the abnormal cell and the type of normal cell from which it arose. Practically, we are restricted to a comparison with neighbouring tissue of the same general type as that from which the abnormal may have arisen. Comparisons of this kind are given in Table II.

Table II. *Comparisons between growths and normal tissue.*

		% on dry weight		Phosphatides		
No.	Tissue	Phosphatides	Free cholesterol	Free cholesterol	% cholesterol bound	% fat in total lipoids
11 A	Carc. kidney	11·70	1·238	9·45	39	54
11 B	Normal kidney	8·04	1·522	5·3	4	45
15 A	Sarc. uterus	8·98	1·667	5·4	37	40
15 B	Uterine muscle	3·77	0·788	4·8	—	45
5 B	Uterine fibromyoma	2·71	0·544	5·0	12	28
5 A	Uterine muscle	3·30	0·749	4·4	12	37
7 A	Uterine fibromyoma	1·45	0·247	5·9	28	37
7 B	Uterine muscle	3·04	0·606	5·0	5	36
10 A	Uterine fibromyoma	1·395	0·320	4·4	?	50
10 B	Uterine muscle	2·97	0·818	3·6	?	51
16 B	Uterine fibromyoma	5·93	0·753	7·9	?	13
16 A	Uterine muscle	5·42	0·936	5·8	0	24

The two malignant tissues are fortunately not admixed with normal tissue; of the four fibromyomata, one is degenerating and one is admixed with normal muscle. In all cases the abnormal tissue shows a higher ratio of phosphatide to free cholesterol than the normal tissue. Malignant tissue shows also a higher phosphatide content than the normal, but for the innocent growths the converse is usually true for both phosphatides and cholesterol. If the data for the fibromyomata and corresponding normal tissues be examined, it will be seen that regularities, which may be extended to the proportion of free fat in the lipoids, emerge only if each fibromyoma is compared with the neighbouring normal muscle removed at the same operation. If fibromyomata and uterine muscle are compared indiscriminately as classes, regularities are much less evident.

This suggests tentatively that abnormal tissue retains much of the character of the normal tissue it arises from; a suggestion in agreement with the well-known pathological fact.

In two cases we have data that may be used to answer in a preliminary way the question whether the composition of normal tissue situated close to a malignant growth differs from the composition of that situated further away. The data are given in Table III. In one case a carcinoma of the breast (2 A)[1]

[1] This specimen contained: healthy growth 6, fibrous tissue 4, leucocytes and necrotic tissue 3, and fat 2 parts.

is imbedded in pectoral muscle, and that near the growth (2 B) may be compared with that situated further away (2 C). In the other case a carcinoma (14 A) is surrounded by leg muscle, neighbouring (14 B) or further away (14 C). The specimens of muscle show no gross difference histologically, but

Table III. *Composition of tissue near a growth.*

	% on dry weight			Phosphatides / Free cholesterol
No.	Phosphatides	Free cholesterol	Total cholesterol	
2 A	5·58	0·827	1·389	6·75
2 B	2·66	0·537	0·717	4·95
2 C	2·13	0·615	0·901	3·5
14 A	4·06	1·053	1·208	3·9
14 B	5·74	1·042	0·843	5·5
14 C	2·83	0·264	0·235	10·7

examination of the figures shows some tendency for the normal muscle close to the growth to be more like the growth in composition than is the more distant muscle. Little more can be said with the limited data available, but the subject merits further investigation.

Previous work.

The results of previous work, some of it carried out by methods of doubtful value, will be briefly considered in relation to our findings.

The phosphatide contents of tissues tend to be higher in malignant tissues than in normal, in nervous tissue and glands than in muscle, and in young tissues than in old. In general, active tissues show higher contents than inactive. We may cite, for instance, the work of Enselme and Enselme [1927], who find more phosphatides in the malignant breast than in the normal gland. Decrease of phosphatide content on passing from young to older tissues is found for the testicle by Koch and Woods [1905], for the bone marrow by Glikin [1907], and the carcase of the young white rat by Sinclair [1930]. Sorg [1929] finds that in three kinds of rabbit muscle, the phosphatide content is greater in muscle of greater endurance capacity. According to Momigliano [1925], during involution of the human corpus luteum the phosphatide content decreases. Data are quoted by Bloor, Okey and Corner [1930] to show that the variation of phosphatide content in different animals parallels the intensity of metabolism; the content is, for instance, higher in the mouse than in man.

Bürger and Schlomka [1928] find that the cholesterol content of human skin decreases with increasing age, and Dam [1931] finds a decrease with age for the newly hatched chick. Burgheim and Joel [1931] find the cholesterol content of malignant growths greater than that of benign growths.

The ratio of phosphatides to cholesterol was found to be higher in a mouse tumour of rapid growth than in one of slow growth by Bullock and Cramer [1913–14]. In the corpus luteum of the sow, according to Bloor, Okey and Corner [1930], the phosphatide content parallels the physiological activity,

while the free cholesterol changes similarly but to a less extent. According to Iwano [1925] the lecithin-cholesterol ratio falls with age in transplanted rat tumours.

It cannot be pretended that indiscriminate comparison of different tissues shows any intelligible relations between lipin contents or lipin ratios and any individual structure or function as yet compared. The data of Osato and Heki [1930] on rabbit tissues may be used to illustrate the point. Their mean figures yield the following ratio of phosphatides to free cholesterol: heart muscle 21, liver 16, skeletal muscle 10·5, spleen 9·1, kidney 8·6, lung 6·7, and blood 3·8. It is understandable that heart muscle shows a higher value than skeletal muscle, but further interpretation is at present impossible. Muscle, liver and kidney have all different chemical functions, and a common factor showing a relation with the lipin ratios is yet to be found.

The relation of cholesterol esters to growth is not yet clear. The author finds much of the cholesterol in malignant tissue to be bound; but in transplanted tumours of mice and rats, according to Bullock and Cramer [1913–14], Iwano [1925], and Bolaffi [1929] there is little bound cholesterol. In placenta, according to the present work, there is little bound cholesterol, while Dam [1929, 1931] finds a large proportion in newly hatched chicks, which decreases in the first few weeks of life. Cholesterol esters accompany regression in the corpus luteum [Bloor, Okey and Corner, 1930; Momigliano, 1925]. However, Onizawa [1929] finds that the bound cholesterol varies more than the free cholesterol in tissues, and other data suggest the same. The conclusion may be due to greater experimental error, but yet it seems likely that cholesterol esters are fairly inert and constitute storage material.

Summary.

A discussion of the physico-chemical properties of lecithin and cholesterol leads to the conclusion that the lecithin-cholesterol ratio of growing cells, and probably other active cells, should be high.

A limited set of analyses of human tissues gives the following results. Pure malignant tissues have a higher phosphatide and cholesterol content, and tend to show a higher phosphatide-cholesterol ratio, than do malignant tissues admixed with normal tissue. Malignant tissues show a high proportion of bound cholesterol. Malignant and benign tumours show a higher phosphatide-cholesterol ratio than do the neighbouring tissues from which they may have arisen. Previous work has shown a definite parallelism in tissues between growth and activity and the phosphatide content.

In conclusion, the author wishes to thank Prof. W. C. M. Lewis for the interest he has taken in this work.

REFERENCES.

Bell (1930). Some aspects of the cancer problem. (London.
Bienenfeld (1912). *Biochem. Z.* **43**, 245.
Bloor, Okey and Corner (1930). *J. Biol. Chem.* **86**, 291.
Bolaffi (1929). *Z. Krebsforsch.* **30**, 170.
Bullock and Cramer (1913–14). *Proc. Roy. Soc. Lond.* B **87**, 236.
Bürger and Schlomka (1928). *Z. ges. exp. Med.* **63**, 105.
Burgheim and Joel (1931). *Klin. Woch.* **10**, 397.
Corran and Lewis (1924). *Biochem. J.* **18**, 1364.
Cramer (1916). *J. Physiol.* **50**, 322.
Dam (1929). *Biochem. Z.* **215**, 468.
—— (1931). *Biochem. Z.* **232**, 269.
Dörle (1923). *Z. ges. exp. Med.* **34**, 406.
Eichholtz (1924). *Biochem. Z.* **151**, 187.
Enselme and Enselme (1927). *Bull. Soc. Chim. Biol.* **9**, 1017.
Glikin (1907). *Biochem. Z.* **4**, 235.
Hagihara (1924). *Biochem. Z.* **144**, 482.
Handovsky, Lohmann and Bosse (1925). *Pflüger's Arch.* **210**, 63.
Iwano (1925). *J. Biochem. (Japan)*, **4**, 481.
Jowett and Lawson (1931). *Biochem. J.* **25**, 1981.
Koch and Woods (1905). *J. Biol. Chem.* **1**, 203.
MacLean and MacLean (1927). Lecithin and allied substances. (Longmans, Green and Co., London.)
Momigliano (1925). *Z. Gynäkol.* **49**, 684.
Moravek (1927). *Arch. Clin. Cancer Research*, **3**, 99.
Okunev (1928). *Biochem. Z.* **198**, 296.
Onizawa (1929). *J. Biochem. (Japan)*, **10**, 409.
Osato and Heki (1930). *J. Biol. Chem.* **87**, 541.
Robertson and Burnett (1913). *J. Exp. Med.* **17**, 344.
Rondoni (1930). *Z. Krebsforsch.* **32**, 416.
Sinclair (1930). *J. Biol. Chem.* **88**, 575.
Sorg (1929). *Z. physiol. Chem.* **182**, 97.
Standenath (1925). *Biochem. Z.* **155**, 245.
Theorell (1930). *Biochem. Z.* **223**, 1.
Tsuneyoshi (1927). *J. Biochem. (Japan)*, **7**, 235.

CCXVI. THE LOSS OF GLUCOSE FROM DRIED PEAS ON SOAKING.

By WINIFRED MARY CLIFFORD.

From the Physiology Department, King's College of Household and Social Science, Campden Hill Road, Kensington, W. 8.

(*Received October 22nd, 1931.*)

THE common treatment of dried peas when used as human food is to subject them to a prolonged soaking so that they may imbibe water and to follow this up by boiling the peas.

Hutchison [1927] says of this process, "...soaking (is) inevitably accompanied by some loss of protein and mineral matter and also of carbohydrates." Similar statements are met with in many cookery books and have given rise to the idea that such a loss is considerable and may cause a decided drop in the food value of the peas.

Masters [1918] investigated the loss incurred by beans on soaking and found that 100 g. soaked in 250 cc. tap or distilled water lost 1·5–2·2 % solids and of this a considerable proportion consisted of nitrogenous extractives and salts. It was decided to carry out similar experiments with peas with regard to the loss of carbohydrate.

At the same time, opportunity was given to investigate the further statement of Hutchison [1927], that "peas do not readily soften in water containing lime...hence the importance of adding soda to hard water in which pulses are to be soaked."

EXPERIMENTAL METHODS.

The loss of carbohydrate from peas when soaked in various solutions was determined as follows.

100 g. of dried peas were placed in 250 cc. of the liquid and kept overnight. In the morning the surplus was strained off through butter-muslin and measured. This fluid, if acid, was neutralised with anhydrous sodium carbonate and estimations of reducing sugar carried out, using Benedict's method. An osazone was made from the specimen in any series giving the strongest reaction, and a heavy crop of glucosazone crystals resulted in every case. Therefore all reducing substances were calculated as glucose. No iodine reaction was obtained with any fluid investigated, and there were only minute traces of any non-reducing sugar giving rise to reducing sugars on hydrolysis. Each fluid gave faint reactions with the protein colour tests, but no differences

were noticed in sugar titrations when carried out before or after removal of the traces of protein.

For experiments on the rate of cooking, the peas when strained from their soaking fluids were loosely tied in butter-muslin and immersed in an open saucepan of boiling water. This water was kept boiling vigorously for 40 minutes when the peas were removed. Peas soaked in distilled water were invariably well cooked in this time, and the others were compared with them.

Experimental results.

A = cc. fluid remaining after soaking (originally 250 cc.).
B = g. glucose in A, *i.e.* glucose lost from 100 g. peas.

Inorganic acids.

Concentration of solution	0		0·015 *N*		0·03 *N*		0·06 *N*		0·125 *N*		0·25 *N*		0·5 *N*	
	cc. A	g. B	cc. A	g. B	cc. A	g. B	cc. A	g. B	cc. A	g. B	cc. A	g. B	cc. A	g. B
HCl	147	0·13	147	0·25	148	0·24	150	0·24	151	0·13	154	0·17	153	0·62
HNO_3	145	0·11	147	0·19	149	0·18	150	0·17	152	0·11	153	0·12	152	0·38
H_3BO_3	144	0·16	145	0·15	145	0·15	145	0·15	145	0·13	—	—	—	—
H_2SO_4	144	0·11	145	0·18	147	0·21	150	0·21	152	0·14	152	0·13	152	0·21
H_3PO_4	135	0·16	138	0·20	140	0·30	143	0·45	146	0·52	148	0·53	150	0·30

Organic acids.

Concentration of solution	0		0·015 *N*		0·03 *N*		0·06 *N*		0·125 *N*		0·25 *N*		0·5 *N*	
	cc.	g.	cc.	g.	cc.	g.	cc.	g.	cc.	g.	cc.	g.	cc.	g.
Acid	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Formic	146	0·12	148	0·41	152	0·54	154	0·60	154	0·60	154	0·59	154	0·43
Acetic	140	0·13	141	0·28	144	0·43	148	0·76	150	0·78	155	0·80	154	0·80
Lactic	143	0·10	144	0·37	144	0·47	150	0·49	152	0·49	158	0·50	160	0·47
Oxalic	134	0·12	140	0·35	142	0·39	148	0·45	150	0·45	150	0·23	154	0·14
Malic	140	0·13	142	0·18	142	0·21	144	0·32	146	0·44	150	0·51	154	0·55
Tartaric	140	0·11	144	0·22	144	0·28	143	0·35	148	0·42	150	0·45	150	0·46
Citric	138	0·12	138	0·18	138	0·25	138	0·31	142	0·38	148	0·45	148	0·42

Of the inorganic acids boric acid alone appears to have no effect. The results with hydrochloric, nitric and sulphuric acids are similar to each other. There is a decrease of water taken up by the peas with increased strength of acid. The sugar lost does not run parallel with this change in absorption, neither does it vary directly with strength of acid. With 0·5 *N* acids most sugar is lost, but with 0·125 *N* only as much as with distilled water. There is another peak of relatively high sugar loss at 0·015 *N*–0·06 *N*. These results suggest that, though with the strongest acid used, hydrolysis of a complex carbohydrate may account for the increased glucose loss, in the lower concentrations some other factor is responsible for the result. This factor is not a simple increase in permeability of the plant cells since with distilled water there is a maximum uptake of water with a minimum of sugar loss.

Phosphoric acid differs from the other inorganic acids in that more sugar is lost from the peas on soaking. This loss is greatest from 0·06 *N* to 0·25 *N* and is considerably decreased at 0·5 *N*. As with the other inorganic acids the amount of liquid taken up by the peas decreases steadily with increasing

strength of acid. The curve obtained on plotting the glucose loss against concentration of acid in the case of phosphoric acid is unlike that obtained from the other inorganic acids, but similar to that obtained from the organic acids.

The results with organic acids are similar to those obtained with the inorganic, but there is a greater glucose loss with each concentration.

The degree of imbibition of liquid by the dry peas is the same as with inorganic acids both quantitatively and qualitatively. These two sets of results again show that glucose removal is not solely a matter of permeability. The most powerful agent in these experiments was acetic acid which at 0·125 *N* removed 0·78 g. glucose from 100 g. peas.

The shape of the curves obtained from these results is unlike that from the inorganic acids, there being no dip after the maximal rise at 0·06 *N* except for a small one in the case of formic acid and a pronounced one with oxalic acid. These two acids give curves similar to that obtained with phosphoric acid. In no case was there anything comparable with the second rise in glucose removal seen with HCl, HNO_3 and H_2SO_4 between 0·25 *N* and 0·5 *N*.

The fall of glucose with the stronger solutions of oxalic acid was unexpected in view of the statement so often put forward in cookery books and by Hutchison [1927] that the presence of calcium causes a decrease in the permeability of peas when soaked. Therefore peas were soaked in an 0·25 *M* solution of $CaCl_2$; this gave 0·14 g. glucose per 100 g. peas and a residual fluid of 137 cc., whilst the control with distilled water gave 0·15 g. glucose in 147 cc. residual fluid. Hence the extra calcium had no effect on loss of glucose, but depressed water imbibition, again showing the independence of the two phenomena.

This result led to further experiments with salts in the soaking liquids.

Alkalis.

Concentration of solution	0		0·015 *N*		0·03 *N*		0·06 *N*		0·125 *N*		0·25 *N*		0·5 *N*	
	cc. A	g. B	cc. A	g. B	cc. A	g. B	cc. A	g. B	cc. A	g. B	cc. A	g. B	cc. A	g. B
NaOH	140	0·16	140	0·11	140	0·08	136	0·07	136	0·07	134	—	130	—
Na_2CO_3	146	0·16	139	0·14	140	0·17	140	0·16	142	0·17	146	0·15	152	—
$NaHCO_3$	136	0·13	138	0·13	140	0·11	140	0·11	143	0·11	150	0·11	154	0·19

Of the alkalis sodium hydroxide gave a small decrease in glucose removal, sodium bicarbonate a still smaller decrease, and sodium carbonate had no effect. At the highest concentration of sodium hydroxide and carbonate it was not possible to estimate the glucose as the Benedict's solution was decomposed.

Two of the alkalis behaved in the same way as the acids in decreasing the amount of water taken up by the peas. This was not so with sodium hydroxide, probably because at concentrations greater than 0·06 *N* the peas in the cold became pulpy and difficult to strain from the liquid.

Salts.

Concentration of solution	0		0·015 *M*		0·03 *M*		0·06 *M*		0·125 *M*		0·25 *M*		0·5 *M*	
	cc. A	g. B	cc. A	g. B	cc. A	g. B	cc. A	g. B	cc. A	g. B	cc. A	g. B	cc. A	g. B
Sodium tartrate	139	0·17	140	0·16	141	0·15	144	0·15	142	0·15	160	0·13	170	0·14
Sodium bitartrate	140	0·14	142	0·21	144	0·22	146	0·32	145	0·36	150	0·47	154	0·53
Na_2SO_4	142	0·14	142	0·14	144	0·14	146	0·14	160	0·14	170	0·11	175	0·09
$NaHSO_4$	142	0·13	148	0·20	150	0·25	152	0·23	154	0·20	158	0·16	160	0·19
Na_3PO_4	136	0·15	136	0·15	138	—	138	—	146	—	153	—	154	—
Na_2HPO_4	136	0·15	138	0·14	140	0·12	142	0·12	144	0·12	146	0·17	—	—
NaH_2PO_4	138	0·17	138	0·17	142	0·17	142	0·16	144	0·17	150	0·17	160	0·18

With the majority of the salts there was no appreciable difference in the amount of glucose removed from 100 g. peas, as compared with that removed by distilled water. The two exceptions were sodium hydrogen tartrate which gave a steady increase with increase of concentration and sodium hydrogen sulphate which gave a result similar to that with sulphuric acid, *viz.* a rise at the lower concentrations tailing off at the higher concentrations.

All the salts behaved like the acids and alkalis in lessening the amount of water taken up by the dried peas. This was specially marked with sodium tartrate and sodium sulphate which when used at a concentration of 0·5 *M* so decreased the imbibition of water that the peas remained wrinkled after 12 hours' soaking, although the salt concentration in the soaking fluid caused them to become as soft as though cooked.

Effect on rate of cooking.

When peas soaked in 0·5 *N* solutions of various organic and inorganic acids were cooked in vigorously boiling water for 45 minutes they became quite soft, but their outer coats remained hard, and therefore they appeared to be less cooked than the control peas which had been soaked in distilled water. This effect was not seen at 0·25 *N* and lower concentrations where the acid-soaked peas behaved exactly as the controls and were well cooked in 40 minutes.

With the alkalis, soaking in 0·25 *N* solutions in the cold rendered the peas as soft as though cooked and they disintegrated to a pulp on being placed in boiling water. Lower concentrations did not affect the time required for cooking. Similar softening in the cold accompanied by disintegration on heating occurred with sodium tartrate and bitartrate, sodium sulphate and bisulphate and the three sodium phosphates at high concentrations. This softening is therefore not due solely to increased alkalinity since some high salt concentrations, even of acid salts, have the same effect.

Peas soaked in 0·25 *M* $CaCl_2$, NaCl, KCl and distilled water gave identical results as to time of cooking and also in glucose removed during 12 hours' soaking.

From these results it appears that so far as carbohydrate loss is concerned, it is immaterial whether peas are soaked in hard or soft water. Further

the addition of the customary "pinch" of bicarbonate does not alter this slight loss or accelerate the cooking of the soaked peas. Its only effect is the usual attainment of a bright green colour by the peas soaked in a weak solution of alkali.

Although the carbohydrate loss incurred on soaking peas is negligible from the point of food value, it is markedly altered by the presence of acids in the steeping fluid. This alteration is not a direct function of p_H since organic acids of the same normality differ markedly amongst themselves in power to extract glucose from peas, and are 2–4 times more powerful in this respect than inorganic acids of the same normality. Any increase of ionic concentration reduces the water imbibed by dried peas.

Hence the increase in glucose loss with acids is not the result of a simple change in permeability of the cell wall, but is probably due to the liberation of glucose from some preformed combination in the plant cells.

SUMMARY.

1. Dried peas on soaking in distilled water for 18–24 hours lose 0·1–0·15 % glucose, *i.e.* about 0·2 % of their total carbohydrate.
2. This loss is unaltered by the addition of alkalis to the soaking fluid.
3. The addition of acids causes a marked rise in loss of glucose.
4. This glucose loss is more marked with organic acids than with inorganic acids.
5. The uptake of water by dried peas is depressed by the addition of acids, alkalis or salts to the soaking fluid.
6. The rate of cooking of peas is unaltered by previously soaking them in acids, alkalis, or salts unless the concentration of alkali or salt is high enough to soften the whole pea in the cold (this would render the pea inedible).

The expenses of these experiments were defrayed by a grant from the Medical Research Council.

REFERENCES.

Hutchison (1927). Food and principles of dietetics, p. 232. (Arnold, London.)
Masters (1918). *Biochem. J.* **12**, 231.

CCXVII. THE ENZYMIC SYNTHESIS OF PROTEINS WITH SPECIAL REFERENCE TO THE ACTION OF PEPSIN.

By DAVID PATON CUTHBERTSON
and SIDNEY LIONEL TOMPSETT.

From the Biochemical Laboratory of the Institute of Pathology of the Royal Infirmary and University of Glasgow.

(*Received October 22nd, 1931.*)

The first enzymic synthesis of protein from substances of lower molecular weight was described by Danilewski, who observed [1886] that, when an extract of stomach was added to a concentrated solution of the products of a peptic digest of protein, a precipitate formed. Okunew [1895] obtained similar results. The phenomenon was further investigated by Sawjalow [1901] who gave the name plastein to the precipitate he obtained. Taylor [1907, 1909] digested protamine sulphate with a glycerol extract of the liver of the Pacific coast clam and, after concentration of the digestion products and further addition of the enzyme, obtained at the end of 5 months' incubation a substance which was identified as protamine sulphate.

Regarding the nature of plastein little was known by the earlier workers. Okunew [1895] stated that his digests contained 2 % more water after plastein formation had taken place. Sawjalow [1901] believed that plastein was a more complex substance than its precursors owing to its greater ease of precipitation by metallic salts. Henriques and Gjaldbäk [1911, 1912] found that there was always a decrease in nitrogen titratable in presence of formaldehyde during plastein formation. They determined the ratio of free amino-nitrogen to total nitrogen of purified specimens of plasteins and found the figures to be comparable with those of the native proteins. Wasteneys and Borsook [1925, 2] do not however consider this ratio to be a fair criterion of the complexity of protein substances.

Morrell, Borsook and Wasteneys [1927] demonstrated that the rate of hydrolysis of a protein by pepsin, as measured by the amount hydrolysed in the first half hour, diminishes as the concentration of protein increases. Wasteneys and Borsook [1925, 2] confined their attention to the action of pepsin upon concentrated peptic digests of egg-albumin. They found that plastein formation did not occur if the concentration of the digests were less than 2 g. of nitrogen per 100 cc. These workers [1925, 4] were also the first accurately to determine the optimum p_H for plastein formation. They ob-

tained the greatest yields of plastein from peptic digests of egg-albumin at p_H 4·0 and found that the yield decreased very rapidly on either side of this value.

The present writers have further examined this problem and have extended the investigations to other proteins.

Analytical methods.

The peptic digests were prepared as follows.

To 1000 cc. of water, 25 g. of protein were added followed by sufficient hydrochloric acid to bring the p_H to 1·7. This mixture was incubated for about 6 days at 37° with 0·5 % pepsin. After incubation the whole was placed in a boiling water-bath for 30 minutes to inactivate the enzyme and when cool, filtered. The filtrate was concentrated *in vacuo* at 40° until it had a concentration of 3–4 g. nitrogen per 100 cc.

The amount of plastein formation was determined by the method of Wasteneys and Borsook [1925, 1]. 5 cc. of the digest were diluted to 25 cc. and nitrogen was determined in a 5 cc. sample. To another 5 cc. sample of the digest 15 cc. water were added, then 2·5 cc. of 20 % trichloroacetic acid and finally the whole was diluted to 25 cc. and mixed. The plastein was filtered off and the nitrogen content of 5 cc. of the filtrate was determined. From these two results the percentage plastein formation was calculated.

Influence of hydrogen ion concentration.

Specimens of concentrated peptic digests of egg-albumin, serum-albumin and caseinogen were divided into a series of 10 cc. portions and the p_H of each roughly adjusted by the addition of either dilute HCl or NaOH so that a range from about p_H 2·0 to 6·5 was obtained. The specimens were adjusted to the same volume by the addition of the requisite amount of water. 1 g. of pepsin and a few drops of toluene were added to each. They were stoppered and incubated at 37° for 3 days, being shaken at intervals to facilitate solution of the pepsin. The amount of plastein formation was then determined. The p_H of each specimen was determined electrometrically after the pepsin had dissolved.

The optimum p_H for plastein formation in all peptic digests of the three proteins examined was slightly over 4·0. This is in agreement with the results obtained by Wasteneys and Borsook [1925, 4] for peptic digests of egg-albumin.

The influence of temperature.

Sawjalow [1901] and Henriques and Gjaldbäk [1912] had observed from qualitative experiments that an increase in temperature led to an increase in plastein formation. Robertson [1909] and Wasteneys and Borsook [1925, 3] demonstrated this quantitatively. Plastein formation does not increase indefinitely with temperature, since pepsin is inactivated above 72°. Wasteneys

and Borsook [1925, 3], controlling the p_H and using concentrated peptic digests of egg-albumin, obtained their maximum yield of plastein at 72°, no plastein being formed at 80°.

This phenomenon has been re-investigated, using concentrated peptic digests of egg-albumin and serum-albumin. The digests were adjusted to p_H 4·0 and, before addition of the enzyme, were allowed to come to the temperature at which the reaction was to be studied. Plastein formation was determined after 24 hours' incubation. The results with both proteins were in entire agreement with those of Wasteneys and Borsook [1925, 3].

The effect of enzyme concentration.

The concentration of the enzyme has a marked influence upon the amount of plastein formation. The data obtained are shown in Table I for egg-albumin and serum-albumin.

Table I.

	Concentration of enzyme (g. per 100 cc.)	% plastein formation
Concentrated peptic digest of egg-albumin	0·1	7·9
Total nitrogen—3·94 g. per 100 cc.	0·5	13·2
p_H—4·0	1·0	17·8
Incubated 3 days at 37°	2·0	20·3
	5·0	24·5
	10·0	27·4
Concentrated peptic digest of serum-albumin	0·1	7·9
Total nitrogen—3·75 g. per 100 cc.	0·2	12·8
p_H—4·0	0·5	13·9
Incubated 3 days at 37°	1·0	18·7
	5·0	21·9
	10·0	24·8

Ratio of decrease of amino- and carboxyl-groups.

So far the only criterion of plastein formation which has been considered, is the property ascribed exclusively to proteins of insolubility in 2 % trichloroacetic acid. Other confirmatory evidence therefore seemed essential.

Henriques and Gjaldbäk [1912] observed a decrease in nitrogen titratable in presence of formaldehyde during plastein formation. Oda [1926] contested the view that there was any decrease in the free amino-nitrogen. A decrease in the free amino-nitrogen alone cannot however be taken as evidence of the formation of a more complex molecule. It must be shown that there is no simultaneous formation of ammonia such as could be explained by a hydrolysis of amino-groups. Wasteneys and Borsook [1925, 5] found that if either peptic digests of egg-albumin, glycine solutions or solutions of glycine and phosphate were incubated at 37° with high concentrations of glucose, a marked decrease in free amino-nitrogen was observed when the mixtures were more alkaline than p_H 7·0, the effect increasing with increasing alkalinity. They were unable to account for this disappearance of free amino-nitrogen as ammonia, urea or cyanic acid. In the case of the peptic digests, this

diminution in the free amino-nitrogen did not involve any formation of more complex products of protein nature. It must be noted that none of these changes was obtained in the region of p_H 4, the optimum for plastein formation by pepsin.

Steudel, Ellinghaus and Gottschalk [1926] and Steudel and Ellinghaus [1927] state that carboxyl groups are liberated five times as fast as amino-groups during hydrolysis of a protein by pepsin. Felix and Harteneck [1927], however, using histone and Weber and Gesenius [1927] using caseinogen have found that during peptic hydrolysis of these proteins, amino- and carboxyl groups are liberated at equal rates. This was confirmed by Waldschmidt-Leitz and Künster [1927] for egg-albumin, caseinogen, gelatin and histone, and by Sørensen and Katschioni-Walther [1928] for caseinogen, gelatin and gliadin. Cannan and Muntwyler [1930] found that the acid- and alkali-binding powers of gelatin increased at equal rates during peptic digestion.

It is to be expected that during synthesis of a protein, a reversal of the above process will take place, namely, that the concentration of the amino- and carboxyl groups will decrease at the same rate. Rona and Chrometzka [1927] found that although there was a reduction in the free amino-nitrogen during plastein formation there were only indefinite variations in the concentrations of the carboxyl groups. They sought to explain the phenomenon of plastein formation as a deamination, caused by the liberation of amino-groups to form ammonia, which they determined in the digests by aeration with sodium hydroxide or baryta at 70°. Such drastic treatment would however presumably lead to ammonia formation so that the results obtained cannot be relied upon. Rona and Oelkers [1928] indeed re-investigated the problem and found that there was an equivalent decrease of amino- and carboxyl groups during plastein formation, indicating that the latter is actually a synthesis.

Experiments were made by the following procedure to elucidate this point.

The reaction of the digest to be examined was adjusted so that upon addition of pepsin to a concentration of 10 % the mixture had p_H 4. 0·5 g. of finely divided pepsin was weighed into each of two 25 cc. volumetric flasks, followed by 5 cc. of digest. The contents of one flask were diluted immediately to 25 cc. and well shaken until all was dissolved, and the amino-nitrogen, carboxyl groups and ammonia were determined; these values were taken as the initial values before plastein formation. A drop of toluene was added to the contents of the second flask, the flask stoppered and incubated at 37°. After 2 days the contents of this flask were diluted to 25 cc., well mixed and the amino-nitrogen, carboxyl groups and ammonia again determined. The nitrogen insoluble in 2 % trichloroacetic acid was determined upon a sample from the second flask.

Amino-nitrogen was determined by the method of Linderström-Lang [1928]. The error due to separation of peptones *etc.* in presence of acetone being overcome by carrying out a second titration in which sufficient 0·1 *N* HCl was added prior to the addition of acetone, so that only 1–2 drops of

0·1 *N* acid were necessary to complete the titration. Using this technique consistent results could always be obtained. A blank determination using 1 cc. of distilled water was always carried out, and this correction was allowed for. For carboxyl groups the method employed was that of Willstätter and Waldschmidt-Leitz [1921].

Ammonia was determined as follows: in an 8 × 1 inch pyrex test-tube 1–5 cc. of the solution to be examined were placed. The contents of the tube were diluted if necessary to about 5 cc. with distilled water. 2 cc. of saturated borax solution were added followed by a few drops of liquid paraffin and a glass bead to prevent bumping. A spiral of galvanised iron wire was placed in the tube. A delivery tube was fitted, the end of which dipped under 2 cc. of 0·05 *N* HCl, contained in a test-tube graduated at 25 cc. The ammonia was distilled over by boiling for 4 minutes over a micro-burner. Frothing was prevented by the use of the liquid paraffin and spiral of iron wire. After the distillation, the delivery tube was disconnected and washed into the receiving tube, the contents of which were diluted to about 20 cc. After cooling, the ammonia was determined colorimetrically by Nessler's reagent.

In distilling peptic digests with borax, ammonia from ammonium salts only is liberated, the borax being too weak an alkali to attack the amino-groups. This was proved experimentally with many amino-acids.

The results of these investigations are shown in Table II. The concentra-

Table II.

Total nitrogen (g. per 100 cc.)	Plastein formation %	Amino-nitrogen (cc. *N* HCl %)	Carboxyl (cc. *N* KOH %)	Decrease in NH_2 and COOH: NH_2 (cc. *N* HCl %)	Decrease in NH_2 and COOH: COOH (cc. *N* KOH %)	Ratio of decrease of $\frac{COOH}{NH_2}$	Concentration of ammonia (mg. N per 100 cc.)	Hours
A. Egg-albumin digest. Incubation 2 days at 37°, p_H 4:								
5·139	—	81·0	115·5	—	—	—	36·3	0
5·157	20·7	74·5	109·5	6·5	6·0	0·93	37·1	48
4·419	—	73·0	107·5	—	—	—	48·3	0
4·421	17·2	65·0	98·7	8·0	8·8	1·1	48·7	48
4·160	—	72·0	80·5	—	—	—	25·4	0
4·089	10·5	61·5	69·0	10·5	11·5	1·09	27·1	48
5·198	—	94·3	128·5	—	—	—	30·3	0
5·211	19·7	80·9	115·5	13·4	13·0	0·97	32·6	48
B. Serum-albumin digest. Incubated 2 days at 37°, p_H 4:								
3·516	—	70·2	120·9	—	—	—	56·3	0
3·522	16·4	64·3	115·4	5·9	5·5	0·94	58·1	48
4·144	—	84·3	158·5	—	—	—	49·7	0
4·098	21·4	74·7	148·7	9·6	9·8	1·02	50·3	48
3·917	—	78·0	144·2	—	—	—	61·3	0
4·012	18·7	66·6	133·3	11·4	10·9	0·96	63·1	48
2·961	—	63·4	110·0	—	—	—	27·4	0
3·012	12·8	55·1	102·5	8·3	7·5	0·90	27·8	48
C. Caseinogen digest. Incubated 2 days 37°, at p_H 4:								
3·722	—	87·5	111·5	—	—	—	42·4	0
3·810	10·2	82·1	106·9	5·4	5·6	1·04	43·1	48
3·019	—	80·3	102·4	—	—	—	34·6	0
2·998	8·6	76·1	97·9	4·2	4·5	1·05	34·8	48
4·526	—	90·7	124·6	—	—	—	45·7	0
4·492	11·4	82·4	116·7	8·3	7·9	0·95	46·0	48
4·259	—	86·6	105·4	—	—	—	41·6	0
4·271	9·6	80·1	99·5	6·5	5·9	0·93	41·2	48

tions of the amino- and carboxyl groups are expressed in terms of cc. *N* acid or alkali respectively per 100 cc. digest. The two flasks containing digest and pepsin used for each experiment always contained slightly different amounts of nitrogen, but the amino-nitrogen, carboxyl and ammonia figures of the first flask have been corrected to correspond to the nitrogen figure of the second flask. The corrections, however, were very small, being almost negligible.

It will be seen from the results that in all cases plastein formation is accompanied by an equivalent decrease in the concentration of amino- and carboxyl groups. It appears therefore that plastein formation involves a peptide linking of these groups. These results further strengthen the view that plastein formation is essentially a synthesis. No appreciable formation of ammonia takes place during plastein formation. The amount of plastein formation as determined by the quantity of nitrogen insoluble in 2 % trichloroacetic acid does not appear, however, to run parallel with the decrease in concentration of the amino- and carboxyl groups. This may be due to the fact that in measuring the former, only one phase of the reaction is being determined. Wasteneys and Borsook [1925, 2] have shown that during plastein formation all the fractions of a protein hydrolysate appear to be involved, there being an increase of certain fractions such as proteoses and peptones from molecules of less complexity.

The effect of the degree of enzymic hydrolysis on the process of re-synthesis.

Sawjalow [1901] concluded that the nearer a peptic digest of protein approaches in complexity to the original protein, the more easily does plastein formation take place. Henriques and Gjaldbäk [1912] and Wasteneys and Borsook [1930] controlling the p_H reached similar conclusions. We have re-investigated this point, using serum-albumin.

A peptic digest of serum-albumin was prepared and at intervals of 5, 10, 20 and finally 30 days portions were removed, the active enzyme destroyed by heat, the unchanged protein filtered off and the filtrate concentrated *in vacuo*. The samples were concentrated to approximately the same degree. Pepsin was added to the concentrated digests to 10 % concentration and the mixtures brought to p_H 4. The amount of plastein formation was determined at intervals. The results were in agreement with the observations of the above workers.

The character of the original protein and its relationship to plastein formation.

The amount of plastein obtained under comparable conditions varies considerably according to the protein from which the digest has been prepared. While peptic digests of egg- and serum-albumins gave the highest yields, peptic digests of caseinogen were found to give much lower yields. Wasteneys and Borsook [1930] found that peptic digests of gliadin gave much smaller yields than peptic digests of egg-albumin. So far no investigator has obtained

plasteins from peptic digests of gelatin. The present writers have similarly failed. Wasteneys and Borsook [1930] have suggested that this failure may be due to the use of acid in preparing gelatin from collagen since Henriques and Gjaldbäk [1911, 1912] found that partial acid or alkaline hydrolysates of proteins yielded no plasteins. With this in view the present writers attempted to form plasteins from a concentrated peptic digest of collagen.

Tendons were first digested with trypsin to hydrolyse proteins other than collagen. The collagen was then washed with water to remove the disintegration products of the foreign proteins. The collagen was digested with pepsin at 37° and p_H 1·7 for several days. The procedure for preparing the concentrated peptic digest of collagen was essentially the same as described earlier in this paper. The concentrated digest of collagen was finally incubated 7 days at p_H 4 with 10 % pepsin, but no evidence of plastein formation was obtained. The results of this experiment do not appear to support the theory advanced by Wasteneys and Borsook to account for their failure to produce plastein from concentrated digests of gelatin.

Properties and composition of the plasteins.

The plasteins prepared from the various peptic digests all gave positive protein biuret tests. They were soluble in strong acids and strong alkalis and were coagulated by boiling in alkaline solution, but not in acid solution. The plasteins were all digested rapidly by pepsin at p_H 1·7 and from their peptic digests, primary proteoses could be salted out by half saturation with ammonium sulphate. The sulphur, nitrogen and phosphorus contents of plasteins from various sources were determined and compared with those of the original native proteins. It will be seen from the data (Table III) that the contents

Table III.

Source from which plastein was prepared	Sample	Composition of plastein S %	N %	P %	Composition of protein from which digest was prepared S %	N %	P %
Egg-albumin	1	1·63	13·41	—	1·62	15·51	—
	2	1·70	13·82	—	—	—	—
	3	1·58	13·56	—	—	—	—
	Average	1·63	13·59				
Serum-albumin	1	1·91	14·01	—	1·93	15·93	—
	2	1·83	13·89	—	—	—	—
	3	2·10	13·76	—	—	—	—
	Average	1·94	13·89				
Caseinogen	1	0·81	14·45	0·11	0·78	15·6	0·81
	2	0·83	14·02	0·16	—	—	—
	3	0·76	14·76	0·21	—	—	—
	Average	0·80	14·41	0·16			

of these elements in the plasteins and the original proteins are of the same order, with the exception of phosphorus which is almost absent from plasteins derived from caseinogen.

Sulphur was determined by Denis's modification of Benedict's method and phosphorus by the direct weighing of the ammonium phosphomolybdate formed by Neumann's reaction.

In Table IV are shown the cystine content and strength of the Molisch α-naphthol tests both in the plasteins and in the proteins from the digests of which they were derived. The method for the determination of cystine was a modification of the method of Folin and Marenzi [1929] and is described in a subsequent note by one of us [Tompsett, 1931].

Table IV.

	Cystine %	Molisch test
Egg-albumin	2·29	+ +
Plastein (egg-albumin digest)	3·07	+ +
Serum-albumin	2·34	+ +
Plastein (serum-albumin digest)	3·84	+ +
Caseinogen	0·38	faintly +
Plastein (caseinogen digest)	1·35	+ +
Pepsin	0·78	+ +

Discussion.

It has been shown that when peptic digests of egg-albumin, serum-albumin and caseinogen are concentrated to about 3–4 g. nitrogen per 100 cc., upon addition of pepsin and incubation at 37°, an apparent synthesis of protein results, the maximum synthesis being obtained at p_H 4.

The amount of protein or plastein as it has been termed is also influenced by the temperature and the concentration of the enzyme.

Plastein is insoluble in 2 % trichloroacetic acid, gives a positive biuret test and its formation is accompanied by an equivalent decrease in the concentrations of amino- and carboxyl groups without any formation of ammonia. It appears then that plastein formation is actually a synthesis of protein from protein decomposition products. The highest yields were obtained from egg-albumin and serum-albumin digests, caseinogen digests giving much smaller yields, while gelatin digests produced no plastein at all.

The facts that Henriques and Gjaldbäk [1911, 1912] have been unable to obtain plasteins by the action of pepsin upon partial acid or alkaline digests of proteins and that these investigators and also Wasteneys and Borsook were unable to prepare plasteins by the action of trypsin upon tryptic digests of proteins are of great interest. It is curious too that the longer pepsin acts upon a protein during the hydrolytic stage, the lower is the yield of plastein from the subsequently concentrated digest. The possibility arises that pepsin can only unite those products of protein decomposition containing certain groupings in their structure. While little information was derived from the determination of the percentage of sulphur, nitrogen and phosphorus in the plasteins, the figures obtained being of the order of those of the native proteins from which the plasteins were derived, more information was derived from

the analyses of the plasteins for cystine and also by the strength of the Molisch test for carbohydrate. In each case the plasteins contained a higher percentage of cystine than the corresponding native protein. Serum- and egg-albumin both gave strong Molisch tests as did their corresponding plasteins, but the plasteins from caseinogen digests all exhibited stronger tests than the original caseinogen which gave only a faint reaction. Doubt has often been expressed as to whether the protein molecule really contains a carbohydrate or not. Recently, however, Rimington [1929, 1931] and Levene and Mori [1929] have isolated carbohydrate complexes from purified proteins which appear to consist of glucosamine and mannose units and are present to the extent of about 2 %. The question arises as to the importance of cystine and the carbohydrate group of the protein molecule in the formation of plastein. It is suggestive that serum- and egg-albumin contain fairly high percentages of cystine and give strong Molisch tests, while caseinogen contains a very low percentage of cystine and gives a faint Molisch test and gelatin gives no Molisch test and contains no cystine.

Summary.

1. The optimum hydrogen ion concentration for the synthesis of plastein from concentrated digests of egg- and serum-albumin and also caseinogen, incubated at 37°, was p_H 4.

2. The amount of plastein formed from egg- and serum-albumin digests increased rapidly with temperature, reaching a maximum at 70°.

3. Increase in the concentration of enzyme increased plastein formation in concentrated peptic digests of egg- and serum-albumin.

4. The ratio of the decrease in the amino- and carboxyl groups during the process of plastein formation from egg- and serum-albumin and also caseinogen digests was 1 : 1.

5. There was no appreciable liberation of ammonia during formation of plastein.

6. The longer a protein was allowed to digest the less was the yield of plastein.

7. Peptic digests of egg- and serum-albumin gave better yields than those of caseinogen. Gelatin yielded no plastein from the products of digestion.

8. Certain properties of the plasteins are described.

We are much indebted to the Carnegie Trust for a grant in aid of this research.

REFERENCES.

Cannan and Muntwyler (1930). *Biochem. J.* **24**, 1012.
Danilewski (1886) quoted by Henriques and Gjaldbäk (1911).
Felix and Harteneck (1927). *Z. physiol. Chem.* **165**, 103.
Folin and Marenzi (1929). *J. Biol. Chem.* **83**, 103.
Henriques and Gjaldbäk (1911). *Z. physiol. Chem.* **71**, 485.
—— —— (1912). *Z. physiol. Chem.* **81**, 439.
Levene and Mori (1929). *J. Biol. Chem.* **84**, 49.
Linderström-Lang (1928). *Z. physiol. Chem.* **174**, 275.
Morrell, Borsook and Wasteneys (1927). *J. Gen. Physiol.* **8**, 601.
Oda (1926). *J. Biochem.* (*Japan*), **6**, 77.
Okunew (1895) quoted by Henriques and Gjaldbäk (1911).
Rimington (1929). *Biochem. J.* **23**, 430.
—— (1931). *Biochem. J.* **25**, 1062.
Robertson (1909). *J. Biol. Chem.* **5**, 393.
Rona and Chrometzka (1927). *Biochem. Z.* **189**, 249.
—— and Oelkers (1928). *Biochem. Z.* **203**, 298.
Sawjalow (1901) quoted by Wasteneys and Borsook (1930).
Sørensen and Katschioni-Walther (1928). *Z. physiol. Chem.* **174**, 251.
Steudel and Ellinghaus (1927). *Z. physiol. Chem.* **166**, 84.
—— —— and Gottschalk (1926). *Z. physiol. Chem.* **154**, 21, 198.
Taylor (1907). *J. Biol. Chem.* **3**, 87.
—— (1909). *J. Biol. Chem.* **5**, 381.
Tompsett (1931). *Biochem. J.* **25**, 2014.
Waldschmidt-Leitz and Künster (1927). *Z. physiol. Chem.* **171**, 70, 290.
Wasteneys and Borsook (1925, 1). *J. Biol. Chem.* **62**, 1.
—— —— (1925, 2). *J. Biol. Chem.* **62**, 15.
—— —— (1925, 3). *J. Biol. Chem.* **62**, 633.
—— —— (1925, 4). *J. Biol. Chem.* **62**, 675.
—— —— (1925, 5). *Biochem. J.* **19**, 1128.
—— —— (1930). *Physiol. Rev.* **10**, 110.
Weber and Gesenius (1927). *Biochem. Z.* **187**, 410, 429.
Willstätter and Waldschmidt-Leitz (1921). *Ber. deutsch. chem. Ges.* **54 B**, 2988.

CCXVIII. A NOTE ON THE DETERMINATION OF CYSTINE IN PROTEINS BY THE METHOD OF FOLIN AND MARENZI.

By SIDNEY LIONEL TOMPSETT.

From the Biochemical Laboratory of the Institute of Pathology of the Royal Infirmary and University of Glasgow.

(*Received October 22nd, 1931.*)

THERE have been many modifications of the original method of Folin and Looney [1922] for the determination of cystine in proteins. Folin and Trimble [1924] made the method more specific for cystine by removing most of the molybdenum compounds from the phosphotungstic acid reagent thereby decreasing its sensitivity towards tryptophan, tyrosine and phenols. Folin and Marenzi [1929, 1] have recently stated that it is necessary to add much more of the reagent than employed by Folin and Looney and also recommend adding the sulphite before the alkali since a smaller amount is required. Folin and Marenzi [1929, 2] have published the details for the preparation of the phosphotungstic reagent free from all traces of molybdenum. Rimington [1930] examining this method found that a turbidity often formed in the standard and the test solutions, and that the intensity of the colour varied appreciably with slight changes in the concentration of sodium carbonate. Folin [1930] recommended the use of urea for the prevention of this turbidity. Rimington [1930] found this to be quite satisfactory for concentrations of sodium carbonate up to 3*N*. Hunter and Eagles [1927] have recommended the use of sodium hydroxide in place of sodium carbonate.

The present writer found that even when urea was used as recommended by Folin [1930] turbidities often occurred in the test and standard solutions in the Folin-Marenzi method. It was found however that a blue colour developed when sodium bicarbonate solutions were used in place of sodium carbonate. The colour obtained was stronger than that with sodium carbonate and turbidity did not occur even after 30 minutes. It was therefore decided to test the possibility of replacing the carbonate by bicarbonate.

The following solutions were used:

(1) phosphotungstic reagent of Folin and Marenzi [1929, 2];
(2) 20 % sodium sulphite;
(3) saturated sodium bicarbonate solution;
(4) standard cystine solution, containing 1 mg. in 1 cc. *N* H_2SO_4.

Into each of three 100 cc. volumetric flasks were measured 2 cc. of standard cystine solution followed by 2 cc. of sulphite solution. After allowing to stand 1 minute to ensure reduction of cystine to cysteine, bicarbonate solution was added as follows: to the first flask 5 cc., to the second 10 cc., and to the third 20 cc. Then 8 cc. of phosphotungstic reagent were added to each, and after 8 minutes they were diluted to 100 cc. and compared in a colorimeter. These three solutions were identical. Similar results were obtained using 1 cc. and 4 cc. of standard respectively, the colour being proportional to the concentration of the cystine in the range tested, *i.e.* 1–4 mg. Turbidities were never encountered so that it was unnecessary to use lithium sulphate or urea. The colours obtained remained stable for at least 30 minutes.

A blank determination was carried out and found to be inappreciable. Solutions of tyrosine, tryptophan, glycine, alanine, glutamic acid, aspartic acid and histidine in concentrations greater than those present in protein hydrolysates were tested by this method. No colour developed apart from the slight colour which develops during a blank determination. No colour was developed by uric acid.

From the results shown in Table I it will be seen that there was no appreciable loss when known amounts of cystine were boiled with 20 cc. of 6*N* H_2SO_4 for 18 hours under the same conditions as used in the hydrolysis of the proteins.

Table I.

Exp.	Initial amount of cystine mg.	Amount of cystine determined after hydrolysis mg.
1	10	10·4
2	20	19·3
3	40	39·4

For the determination of cystine in proteins, 1–5 g. of protein were boiled with 20 cc. of 6*N* H_2SO_4 in a 300 cc. Kjeldahl flask under a reflux condenser for 18 hours. After cooling, the hydrolysate was filtered, washed into a 100 cc. volumetric flask and made up to 100 cc. A volume of 5–10 cc. containing about 1–4 mg. cystine was measured into a 100 cc. volumetric flask, followed by 2 cc. of sulphite solution. After 1 minute 20 cc. of saturated sodium bicarbonate solution were added and then 8 cc. of phosphotungstic reagent. After 8 minutes the mixture was diluted to 100 cc. and, after mixing, compared with a suitable standard prepared in the same way.

The cystine contents of a number of proteins were determined and the results are shown in Table II.

Table II.

Protein	Cystine (%)
Gelatin (B.D.H.)	Trace
Caseinogen (B.D.H.)	0·38, 0·41, 0·34 (average 0·38)
Edestin (B.D.H.)	1·46, 1·42, 1·48 (average 1·45)
Egg-albumin (Kahlbaum)	2·29, 2·29, 2·35 (average 2·31)
Serum-albumin (Kahlbaum)	2·29, 2·26, 2·34 (average 2·30)

SUMMARY.

A modification of the Folin-Marenzi method for the determination of cystine in proteins is described in which a solution of sodium bicarbonate takes the place of the carbonate used in the original method.

The colour produced in the presence of bicarbonate is uninfluenced by the concentration of this substance and turbidity has never been experienced.

The cystine contents of certain proteins have been determined.

REFERENCES.

Folin (1930). *J. Biol. Chem.* **86**, 179.
—— and Looney (1922). *J. Biol. Chem.* **51**, 421.
—— and Marenzi (1929, 1). *J. Biol. Chem.* **83**, 103.
—— —— (1929, 2). *J. Biol. Chem.* **83**, 109.
—— and Trimble (1924). *J. Biol. Chem.* **60**, 473.
Hunter and Eagles (1927). *J. Biol. Chem.* **72**, 177.
Rimington (1930). *Biochem. J.* **24**, 1114.

CCXIX. THE HEMICELLULOSES OF THE WOOD OF ENGLISH OAK.

I. THE EFFECT OF THE DRYING OF WOOD ON THE YIELD AND COMPOSITION OF HEMICELLULOSE A.

By MARGARET HELENA O'DWYER.

From the Section of Chemistry, Forest Products Research Laboratory, Princes Risborough, Bucks.

(*Received October 28th, 1931.*)

Introduction.

In two recent papers Campbell and Booth [1930, 1931] have drawn attention to the chemical changes which take place in wood during various drying treatments, and have shown that these changes are associated principally with the furfuraldehyde-yielding complexes in both the hardwood and softwood examined. Since the hemicelluloses previously investigated [O'Dwyer, 1923, 1926, 1928] consisted largely of such complexes and exhibited marked instability towards heat, it has been decided to examine the general chemical character of the hemicelluloses of the wood of English oak with particular reference in the first instance to the effect on these substances of various methods of drying the original wood. The material used was cut from English oak wood of approximately 40 years' growth from the same locality as that used by Campbell and Booth [1930]. Hemicellulose A was obtained from samples of the wood (*a*) in the green state, (*b*) after air-drying, (*c*) after kiln-drying, and (*d*) after drying to constant weight in an oven at 100°.

Experimental.

I. *Preparation of material.*

(*a*) *From green wood.* A portion of each of the sapwood and heartwood from the original wood was converted to sawdust as soon as possible after felling, and the preparation of hemicelluloses commenced without delay. After conversion, the sapwood attained a moisture content of 25·0 % and the heartwood 27·5 %.

(*b*) *From air-dried wood.* A portion of the original wood was converted to 1-inch boards, piled, and air-dried for several months. Samples of sawdust were prepared from sapwood and heartwood as before. The sawdust was

screened in each case and the 60–80 mesh material was reserved for the preparation of the hemicelluloses. After conversion the moisture content of the sapwood was 12·28 %, and that of the heartwood 12·82 %.

(*c*) *From kiln-dried wood.*

1. *Mild treatment.* A third portion of the original wood was converted to 1-inch boards and kiln-dried according to the following schedule.

i. *Schedule employed in mild kiln-drying treatment.*

Temperature ° C.	Humidity %	Duration of treatment (days)	Total time (days)	Assumed moisture content %
35·0	85	5	5	60–40
35·0	80	2	7	40–35
37·8	75	1	8	35–30
37·8	65	2	10	30–25
40·6	60	4	14	25–15
40·6	60	3	21	10

The rate of initial warming of the boards was 3° per hour at a constant humidity of 85 %. The moisture content after conversion to 60–80 mesh sawdust was, sapwood 10 %, heartwood 11·37 %. A slight darkening of the wood occurred as a result of the seasoning.

2. Several 1-inch boards were subjected to a comparatively drastic kiln-drying treatment according to the following schedule.

ii. *Schedule employed in drastic kiln-drying treatment.*

Temperature ° C.	Humidity %	Duration of treatment (days)	Total time (days)	Assumed moisture content %
46·1	80	2½	2½	60–40
46·1	75	1	3½	40–35
46·1	70	1	4½	35–30
46·1	65	1	5½	30–25
46·1	60	1	6½	25–20
48·9	55	2	8½	20–15
51·7	50	2	10½	15–10
54·4	50	2	12½	10

The rate of initial warming of the boards was 5° per hour at a constant humidity of 80 %. A decided darkening of the wood was observed after this treatment. The moisture content after conversion to 60–80 mesh sawdust was, sapwood 9 %, heartwood 10·9 %.

(*d*) *From oven-dried wood.* Samples of sawdust prepared from the sapwood and heartwood of the original wood were placed on trays and dried to a constant weight at 100°. This required about 24 hours for each batch.

The sawdust was much darker in colour than that obtained from green wood. The moisture contents after preparation of 60–80 mesh material were, sapwood 7·2 %, heartwood 9·3 %.

II. *Preparation of hemicelluloses.*

The method described for the preparation of these substances from beech wood [O'Dwyer, 1926, 1928] was followed in the present instance, amounts of 60–80 mesh wood flour of from 500 to 1000 g. being employed. The addition of lime water to the sodium hydroxide extract was, however, omitted, the first extraction for 4 hours under a mechanical stirrer with 0·2 % sodium hydroxide being followed by a second, lasting 48 hours, which removed the greater part of hemicellulose B. Hemicellulose A, which it has been found possible to separate incompletely into two different fractions, was removed by three subsequent extractions of the wood with 4 % sodium hydroxide for 48 hours under a stirrer at room temperature. Ground-glass filters were used throughout the work except in the case of the sawdust, which was filtered directly on to the Büchner, no cloth or filter-paper being employed.

The amounts of material extracted with hot water and sodium hydroxide respectively were estimated as percentages of the oven-dried weight of the appropriate wood samples, and furfuraldehyde was determined by the phloroglucinol method in each hemicellulose fraction and wood residue. The analytical results are given in Table I.

Table I. *The relationship between the respective pentosan contents of oak wood and of the hemicellulose derived therefrom before and after various drying treatments.*

(Results expressed as percentages of the oven-dried weight of the respective wood samples.)

	Green		Air-dried		Kiln-dried (mild)		Kiln-dried (drastic)		Oven-dried		
	Sap	Heart	Sap	Heart	Sap	Heart	Sap	Heart	Sap	Heart	Remarks
Hot water extract	6·8	10·2	9·1	12·7	10·6	11·0	11·2	9·0	7·0	9·1	—
Total NaOH extract	15·6	14·8	13·4	12·6	16·8	18·6	20·2	25·7	22·7	22·5	—
Yield of hemicellulose A	10·0	7·3	8·5	6·8	7·0	7·8	6·5	5·5	5·8	3·0	—
Total pentosan content of wood before extraction	24·75	24·88	23·76	23·42	23·74	23·11	—	—	23·61	23·78	Data obtained by Campbell and Booth [1930] for oak wood from the same locality
Pentosan content of hemicellulose A	8·73	6·28	6·55	6·34	5·39	6·93	4·43	5·32	4·85	2·82	—
Total pentosan content of wood after extraction	12·9	12·0	13·3	12·9	12·7	12·2	12·8	11·95	12·9	12·4	—

Tests for methyl alcohol.

As the hemicellulose B isolated from wood on previous occasions appeared to bear a strong resemblance to the pectic substances [O'Dwyer, 1926], the various sodium hydroxide extracts from oak were submitted to the test for methyl alcohol described by Schryver and Wood [1920] in order to ascertain whether de-esterification had taken place under conditions in which it would occur in the case of these substances [Branfoot, 1929].

Table II shows the results obtained, the appearance of the pink colour being taken as an indication of the presence of methyl alcohol.

Table II. *Tests for methyl alcohol in sodium hydroxide extracts of oak wood after various drying treatments.*

	Green		Air-dried		Kiln-dried (mild)		Kiln-dried (drastic)		Oven-dried	
	Sap	Heart	Sap	Heart	Sap	Heart	Sap	Heart	Sap	Heart
1st extraction 0·2 % NaOH	++	+	+	+	+	+	−	+	−	−
2nd extraction 0·2 % NaOH	−	−	−	−	−	−	−	−	−	−
4 % NaOH extraction	−	−	−	−	−	−	+	+	++	++

\+ indicates that test is positive for methyl alcohol.
++ indicates that test is strongly positive for methyl alcohol.
− indicates that test is negative for methyl alcohol.

Discussion of results.

As will be seen from Table I, the method of drying oak wood appears to influence both the yield and the constitution of hemicellulose A. In the first place, although the percentage amount of material extracted from the wood during the preparation of the hemicelluloses increases with increasingly drastic methods of drying, the yield of hemicellulose A actually becomes less. The yield from oven-dried wood is approximately half that obtained from the green material.

Owing to the colloidal nature of the hemicelluloses a considerable amount of loss occurs during their preparation. This is most marked in the case of the green wood, so that the disparity shown in Table I between the yields of hemicellulose A after the various methods of drying is in reality greater than would appear from the figures given. The furfuraldehyde-yielding complexes have been considered as pentosan at this stage of the work, but further study of these complexes will probably show them to consist, to some extent, of uronic acid anhydrides, as in the case of beech wood [O'Dwyer, 1926]. Hemicellulose A from oak heartwood appears to be somewhat less soluble in 4 % NaOH than that from the sapwood, as the latter could be almost completely extracted in one operation. According to Anderson [1931] higher yields of hemicellulose can be obtained from the wood of birch and spruce by extraction with 5 % NaOH in the cold, followed by an extraction with 7 % NaOH on a boiling water-bath, but it is certain that these extracts contain a high proportion of wood components other than hemicelluloses.

After drastic kiln-drying and oven-drying of oak wood, the greater part of the substances other than the hemicelluloses extracted by sodium hydroxide from the wood appeared to combine with the sodium hydroxide to form a salt or salts, not removable by dialysis, as these substances, when dry, contained a high percentage of ash which was largely composed of sodium carbonate. This occurred to a much less extent both in air-dried wood and in that submitted to a mild kiln-drying treatment. It was also noted that under

the more drastic conditions of drying the addition of a much smaller amount of acetic acid was necessary for the neutralisation of the sodium hydroxide extracts and the consequent precipitation of the hemicelluloses. In the case of the green wood, by far the greater part of the acetic acid is recoverable as sodium acetate. It is not improbable that the hemicelluloses combine to some extent with the sodium hydroxide in accordance with the suggestion by Neale [1931] that certain hydroxyl groups of carbohydrates may exhibit weakly acidic properties. This property of these groups has long been recognised by workers on the sugars. It is certain, however, that the addition of acetic acid breaks down any such union since the ash content of hemicellulose A is under 2 % and has been identified as silica with traces of phosphorus.

The presence of methyl alcohol in the sodium hydroxide extracts from which hemicellulose A was obtained would appear to support previous suggestions [O'Dwyer, 1926, 1928] that the hemicelluloses may be transitional products in the transformation of pectin to lignin in the plant. Since methyl alcohol is present in the more concentrated alkali extracts obtained from kiln-dried and oven-dried wood (Table II) it is conceivable that during drying under comparatively severe conditions demethylation is carried further. This is borne out in the work of Campbell and Booth [1930] where it is shown that the methoxyl content of kiln- and oven-dried wood is lower than that of either green or air-dried wood. It will be interesting to note whether the percentage amount of hemicellulose B increases as a result of severe drying conditions, or whether other substances more akin to lignin are formed. Campbell and Booth [1930, 1931] have established an apparent increase in the lignin content of wood during drying, and Klason [1930] suggests that the pentoses, and of these especially xylose, may be regarded as the parent substance of lignin, and further that the pentoses are partly methylated in the leaves of plants.

From the foregoing considerations it is to be concluded that during the drying of timber certain definite chemical changes take place in the hemicelluloses. With increasingly drastic conditions of drying the total alkali-solubility of the wood increases and the yield of hemicellulose A decreases, and thus it is apparent that the hemicellulose A of green wood is transformed into a substance or substances of a different nature as wood dries. The full significance of this transformation may well be far-reaching, and a more detailed investigation of it is in progress.

Summary.

1. Hemicellulose A has been prepared from the sapwood and heartwood of English oak before and after various methods of drying.

2. With increasingly severe conditions of drying, the hot water and sodium hydroxide extracts of wood increase in amount, and the yields of hemicellulose A decrease.

3. It is suggested that under drastic drying conditions hemicellulose A is altered in constitution and gives rise to decomposition products which have not been identified.

The author wishes to express her indebtedness to Mr R. A. G. Knight of the Seasoning Section, Forest Products Research Laboratory, for carrying out the drying treatment, to the late Prof. Perkin and to Prof. Robinson for facilities afforded in the Dyson Perrins Laboratory, Oxford, to Mr W. G. Campbell of the Chemistry Section, Forest Products Research Laboratory, for many helpful suggestions, to Sir James Irvine for criticising the manuscript, and to Mr R. S. Pearson, Director of Forest Products Research for permission to publish these results.

REFERENCES.

Anderson (1931). *J. Biol. Chem.* **41**, 559.
Branfoot (1929). *Food Investigation Special Report No.* 33 (*D.S.I.R.*), pp. 36–39.
Campbell and Booth (1930). *Biochem. J.* **24**, 641.
—— —— (1931). *Biochem. J.* **25**, 756.
Klason (1930). *Ber. deutsch. chem. Ges.* **63 B**, 1548.
Neale (1931). *J. Soc. Chem. Ind.* **50**, 117 T.
O'Dwyer (1923). *Biochem. J.* **17**, 501.
—— (1926). *Biochem. J.* **19**, 656.
—— (1928). *Biochem. J.* **22**, 381.
Schryver and Wood (1920). *Analyst*, **45**, 164.

CCXX. THE CHEMISTRY OF THE WHITE ROTS OF WOOD.

II. THE EFFECT ON WOOD SUBSTANCE OF *ARMILLARIA MELLEA* (VAHL.) FR., *POLYPORUS HISPIDUS* (BULL.) FR., AND *STEREUM HIRSUTUM* FR.

By WILLIAM GEORGE CAMPBELL.

From the Section of Chemistry, Forest Products Research Laboratory, Princes Risborough, Bucks.

(*Received October 28th, 1931.*)

In a previous communication [Campbell, 1930] it has been pointed out that comparatively little experimental work has been done on the chemistry of the particular type of wood decay in which lignin is decomposed, and further, that it is unknown whether such decomposition is invariably accompanied by simultaneous decomposition of the wood carbohydrates. Analytical data were obtained, however, which showed clearly that in the decay of wood by *Polystictus versicolor* (Linn.) Fr. carbohydrates are depleted as well as lignin. Falck [1927] has concluded that in wood decayed by *Fomes annosus* cellulose is decomposed in the advanced stages. In the present investigation the study of the so-called white rots has been continued by examining the respective effects of *Armillaria mellea* (Vahl.) Fr. on beech wood, *Polyporus hispidus* (Bull.) Fr. on ash heartwood, and *Stereum hirsutum* Fr. on oak sapwood.

Experimental.

(1) The experimental procedure and analytical methods were the same as those employed in the previous work. Each sample of 60–80 mesh wood flour of known composition was first sterilised, then inoculated with the appropriate fungus and incubated at 20°. After decay had proceeded to the required stage, the wood residues were washed with cold water until free from acid, dried and sterilised at 105°. The comparative effects of 1 % sodium hydroxide on decayed and sound wood respectively were examined only in the case of beech wood. All analytical results are expressed as percentages by weight of the oven-dry original wood, except where it is stated otherwise.

(2) It was noted that oak sapwood decayed by *Stereum hirsutum* was much lighter in colour than the original sound wood, and furthermore, that in various localities the decayed residue was almost pure white in colour. As a rule these white patches were quite small, but in one culture flask there occurred an unusually large patch of white residue.

Table I. *The effect of 1 % NaOH on beech wood before and after decay by* Armillaria mellea.

	Original wood	Decayed wood	Original wood after alkali-extraction	Decayed wood after alkali-extraction
Duration of decay	—	16 months	—	—
Loss due to decay *plus* cold water washing	—	8·66	—	—
Cold water-soluble	0·64	0·17	—	—
Hot water-soluble	1·65	0·89	—	—
1 % NaOH-soluble	16·63	15·88	—	—
Cellulose	57·49	50·84	58·67	50·10
Lignin	23·79	24·00	19·79	18·42
Pentosans in cellulose	15·50	12·62	18·42	14·65
Pentosans not in cellulose	11·45	11·22	0·60	4·98
Methoxyl content	6·33	5·29	4·81	4·27

By carefully cutting the flask it was possible to remove the greater part of this white material. It was found to have retained the cellular structure of the original wood flour, and when dried to constant weight *in vacuo* over $CaCl_2$ and P_2O_5 it weighed 0·1668 g. After extraction with alcohol-benzene, a standard lignin determination was applied, using the appropriate volume of 72 % H_2SO_4. The lignin content of the sample was 0·0212 or 12·5 %. For

Table II. *Analysis of 60–80 mesh oak sapwood and ash heartwood before and after decay by* Stereum hirsutum *and* Polyporus hispidus *respectively.*

	S. hirsutum		*P. hispidus*	
	Original wood	Decayed wood	Original wood	Decayed wood
Duration of decay	—	13 months	—	12 months
Loss due to decay *plus* cold water washing	—	20·16	—	11·3
Cold water-soluble	2·97	1·40	1·38	0·90
Hot water-soluble	5·02	3·77	3·02	2·32
1 % NaOH soluble	18·66	18·64	21·13	19·45
Cellulose	58·81	48·99	59·39	54·14
Lignin	21·51	15·66	20·30	17·30
Pentosans in cellulose	15·79	11·26	14·63	12·24
Pentosans not in cellulose	8·71	8·13	10·15	10·16
Methoxyl content	5·80	4·05	5·50	4·69

comparative purposes a lignin determination was applied to a small sample of decayed wood taken from the immediate vicinity of the white patch. 0·3408 g. of decayed wood contained 0·0698 g. or 20·48 % of lignin. While the white material was loosely packed together, the surrounding pale brown material was relatively compact, so that a complete separation was possible. During drying, the intensely white colour of the former was observed to change gradually to a pale brown of lighter tint than that of the latter.

Discussion of results.

It is generally accepted that *Polyporus hispidus* and *Stereum hirsutum* cause white rot in wood in the sense that the decayed wood assumes a lighter colour than the original sound wood, and that frequently the decayed material contains patches or pockets of a white material resembling cellulose in

appearance. This opinion has been confirmed in the experiments described above and definite white patches were observed in the case of the decay of oak sapwood by *Stereum hirsutum*. There appears to be some doubt, however, as to whether the decay of wood by *Armillaria mellea* can truly be described as a white rot. In this case the decayed wood was not markedly lighter in colour than the sound wood, despite the fact that Hubert [1924] has described the decay as a white rot. The data in Table I reveal however, that, at the stage of decomposition examined, *A. mellea* causes a type of decay whose chemical effect in wood has not hitherto been described in detail. It can be seen that after 16 months' decay the loss of 8·66 % of the oven-dry weight of the original wood is almost wholly accounted for by cellulose depletion. Of the pentosans, only those associated with the cellulose are attacked, and lignin appears to have been unaffected by the fungus. Thus far, the decay bears a close resemblance in effect to the several brown rots which have been described from time to time, but as brown rots in general have been shown to cause a marked increase in the alkali-solubility of the residual wood substance, the decay caused by *A. mellea* cannot be classified with them, for in this case no marked increase in alkali-solubility of the wood residue takes place. Undoubtedly had part of the water-soluble material not been removed from the decayed wood its alkali-solubility would have been greater than that recorded in Table I, but, at the same time, a tendency is here disclosed towards the reduction of the total alkali-solubility of the major constituents as decay proceeds. The same tendency has been observed in the white rot caused by *Polystictus versicolor* [Campbell, 1930] and the analytical data (Table I) reveal that its cause is to be sought in the marked reduction during decay in the alkali-solubility of the pentosans not in the cellulose. Thus even in the absence of analytical data to show whether lignin is in fact decomposed at a later stage, it is apparent that at the stage examined, decay by *A. mellea* possesses chemical characteristics of both brown and white rots, but that in view of the effect on the alkali-solubility of the decayed residue, it may, with reason, be described as a variety of white rot.

With regard to the respective effects on wood substance of *Stereum hirsutum* and *Polyporus hispidus*, it can be seen from the data in Table II that these are essentially the same, despite the fact that in the former case the loss in weight due to decay is approximately twice as great as in the latter. Lignin is decomposed, but in each case by far the greater proportion of the total loss in weight is accounted for by the depletion of the cellulose and its associated pentosans. The percentage amount of pentosans not in the cellulose remains practically constant throughout the decay, and the fall in the methoxyl content is much less than that of the lignin content. Finally, this type of decay does not cause a marked increase in the alkali-solubility of the principal components of wood substance, and it is probable that this fact can be ascribed to the same causes which are operative in the decay of wood by *Polystictus versicolor* and *Armillaria mellea*.

Consideration of the chemical composition of the patches of white material produced during the decay of oak sapwood by Stereum hirsutum.

It has long been considered by biologists that in certain white rots of wood, where patches or pockets of a white material are found to occur, the fungi concerned have a selective action on lignin, and that the white residue consists entirely of cellulose. This conclusion has been based primarily on the reaction of this material to the so-called cellulose stains, but, so far as is known, no purely chemical test has hitherto been applied to it. This has undoubtedly been due to the fact that the white patches in question are usually small, and that the isolation of samples sufficiently large for analysis has been a matter of difficulty. In the experiments recorded above, the samples were too small for more than one analytical determination in each case, but it was considered that lignin determinations would be comparatively easy to apply, and that, in spite of some degree of experimental error, they would provide a definite answer to the questions as to whether the white patch consisted entirely of cellulose, and whether the fungus had had a marked selective action on lignin.

In the first place, it is apparent that the white material still contains lignin, although its percentage amount cannot be directly compared with the lignin content of the original wood, since the loss in weight due to decay is not known. It may safely be deduced indirectly however that the portion of the original wood which gave rise to the white material must have lost considerably more than 20 % of its oven-dry weight before this residue attained a lignin content of 12·5 %, and, since the lignin content of the original wood (Table II) was 21·51 %, it is obvious that wood components other than lignin—and of these chiefly the cellulose—must have been decomposed. From the fact that the lignin content of the material immediately contiguous to the white patch is approximately the same as the actual lignin content of the decayed wood in Table II, it must be deduced that decay has not been confined to the material which ultimately became white.

Conclusions and summary.

From the above considerations it is to be concluded that the three examples of white rots examined have only one feature in common, and that this is shared by the white rot caused by *Polystictus versicolor*. In every case there is no marked increase in the total alkali-solubility of the major components of wood substance and in the case of *Polystictus versicolor* and *Armillaria mellea* this has been proved to be due to the fact that one component—namely the pentosans not in the cellulose—becomes less soluble in 1 % NaOH as decay proceeds. The white rots in question are thus sharply differentiated from the brown rots, for it has been found in some of the latter, that even in very advanced stages of decay the residual wood substance is much more soluble in alkali than the original sound wood. While it has been established

that the detailed chemical effect on wood substance of both *Polyporus hispidus* and *Stereum hirsutum* is essentially similar in all respects, this effect can in turn be differentiated from that of *Polystictus versicolor* when the manner in which pentosans are decomposed is considered. *Polystictus versicolor* attacks the pentosans not in the cellulose to a greater extent than the pentosans which are associated with the cellulose, whereas *Polyporus hispidus* and *Stereum hirsutum* attack the pentosans in the cellulose and have little or no effect on the pentosans not in the cellulose, at the stages of decay which were examined. In view of the effect on the alkali-solubility of wood substance of the decay caused by *Armillaria mellea*, and the fact that the decayed wood possesses to some extent the visual characteristics of a white rot, it must be concluded that in this type of rot a stage can exist in which lignin is apparently unaffected by the fungus.

In the case of the "pocket" rot caused by *Stereum hirsutum*, it is to be concluded that this fungus cannot be credited with a selective action on lignin. The white patches referred to above represent localities in a decaying mass in which decomposition has progressed to a greater extent than in the surrounding material. In these localities cellulose is decomposed as well as lignin, and even after the development of the white colour some lignin remains.

The author desires to express his indebtedness to Messrs K. St G. Cartwright and W. P. K. Findlay for carrying out the inoculations, to Sir James Irvine for criticising the manuscript, and to Mr R. S. Pearson, Director, Forest Products Research, for permission to publish these results.

REFERENCES.

Campbell (1930). *Biochem. J.* **24**, 1235.
Falck (1927). *Ber. deutsch. chem. Ges.* **60**, 225.
Hubert (1924). *J. Agric. Res.* **29**, 551.

CCXXI. FURTHER OBSERVATIONS ON THE OXIDATION OF LACTIC ACID BY BRAIN TISSUE.

By CHARLES AMOS ASHFORD[1]
and ERIC GORDON HOLMES.

From the Biochemical and Pharmacological Laboratories, Cambridge.

(*Received October 30th, 1931.*)

In an earlier communication [Holmes and Ashford, 1930], we reported experiments bearing upon the question of the "Meyerhof cycle" in brain tissue. If lactate is added to brain tissue *in vitro*, and the system thoroughly oxygenated by shaking in a Barcroft's apparatus, or otherwise, it is easy to show that the amount of such added lactate which disappears is far greater than can be accounted for by the additional oxygen taken up by reason of the presence of the lactate. The relationship $\frac{\text{extra lactic acid disappearing}}{\text{lactic acid equivalent of extra } O_2}$ (the "Meyerhof quotient") has a value of about 3·0. We did not succeed in demonstrating any synthesis of carbohydrate; and such a synthesis is required by the hypothesis advanced by Meyerhof, Warburg and their collaborators.

In this paper, experiments are described which substantiate the validity of our previous findings and amplify them in certain important particulars. In the first place, the experiments reported in our first communication on this subject were all performed in a medium which was buffered by phosphate. The experiments have been repeated, therefore, using the CO_2-bicarbonate buffer, but avoiding the manometric method of Warburg, which involves working with very small amounts of tissue. We have investigated more fully the respiratory quotient of the tissue, both in the presence and in the absence of added lactate, and we have endeavoured to trace the fate of the added lactate which disappears.

Section I.

Experiments carried out with bicarbonate-CO_2 buffers.

Since we desired to follow the disappearance of lactic acid by chemical, and not by indirect manometric methods, it was necessary for us to work on a fairly large scale, and to employ at least 2 g. of tissue. At the suggestion of Prof. J. B. S. Haldane, we decided to estimate the oxygen consumption by means of the gas analysis technique.

[1] Beit Memorial Research Fellow.

For this purpose two flasks with wide necks, and of approximately 50 cc. capacity were obtained and arranged as shown in Fig. 1. The flask and attachments were carried on a wooden carriage, which was so shaped that it could be clamped into the mounting of the Barcroft thermostat and shaken like an ordinary Barcroft manometer. The neck of the flask was marked with a diamond at the point reached by the lower end of the rubber bung, and the volume of the whole apparatus, with tubes in place, accurately determined. The apparatus was made and used in duplicate.

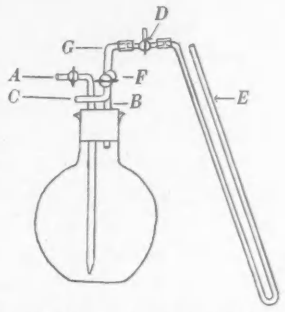


Fig. 1. Diagram (not to scale) of flask with stopper in place. Wooden carriage omitted. *A*. Gas entrance tube. *B*. Tube through which gas escapes during filling, and through which samples are subsequently withdrawn. *C*. Tube through which mercury is expelled during taking of sample. *D*. Three-way tap by means of which flask may be connected to air or manometer. *E*. Mercury manometer. *F*. Two-way tap enabling *B* or *C* to be connected to *G*. All glass tubes and taps are of 1 mm. capillary tubing.

The experimental procedure was as follows.

2 g. of chopped (rabbit's) brain tissue were weighed out, and placed in each flask. 5·0 cc. of Ringer's solution containing 0·025 *M* sodium bicarbonate [Warburg, 1926] was placed in one flask (*OP*) and 5 cc. of a similar bicarbonate Ringer, containing in addition 0·5 % lithium lactate, was added to the other (*OL*). The mass of tissue was broken up with forceps, the stoppers put in place, and the bottles were placed in the carriages. About 4 litres of a CO_2/O_2 (approx. 5 % CO_2 in O_2) gas mixture were bubbled through both bottles, which were arranged in series. Tap *A* was then closed, tap *D* turned so as to connect the bottles to the manometers, and the bottles were placed in the thermostat at 37° and shaken for a few minutes, by which time the manometers had become steady. Tap *D* was then opened to the air for a few moments; taps *D* and *F* were then closed, and the apparatus was shaken at 37° for the required time, usually for 3 hours.

At the end of this period, the bottles were removed, and placed, in their carriages, in a tank of water at room temperature which was stirred by a brisk stream of air bubbles. The manometers were connected to the bottles by turning taps *F* and *D*, and readings of the pressure inside the bottles were taken at intervals, until the manometers became steady. Tap *F* was then closed, and the bottles were removed from their carriages, and immersed in a tall jar of water. They were connected with gas-sampling tubes filled with mercury, and samples withdrawn in the usual way, the dead space in *G* and the connecting tubes being filled with mercury by gravity, the mercury escaping through *C*. The samples were then analysed in a Haldane apparatus [Haldane, 1920], by the standard technique for oxygen-rich mixtures (*i.e.* after preliminary dilution with nitrogen in the gas burette).

In this way sufficient data were obtained to calculate the initial and final volumes of dry gas at N.T.P., and the initial and final percentage composition.

The initial percentage composition was determined in a sample taken direct from the aspirator, where the gas mixture was stored over saturated $CaCl_2$, immediately after the bottles had been filled.

The initial volumes were those of the bottles (already determined) corrected for barometric pressure, temperature (37°), and the tension of aqueous vapour at 37°. The final volumes were those of the bottles, with the following corrections.

(1) Temperature (that of the tank in which they were immersed after removal from the thermostat).

(2) Tension of aqueous vapour at that temperature.

(3) Pressure. This was that of the barometer, *minus* the pressure difference recorded by the manometer. To obtain the latter, the manometer reading had to be multiplied by $\cos\theta$ where θ was the angle of inclination of the manometer to the vertical.

No correction was made for the solubility of O_2 in water at room temperature.

From these data, the amounts of oxygen and CO_2 present in the gas phase in the bottles at the beginning and end of the experiment were calculated, the difference in the values found for oxygen representing the oxygen consumption of the tissues.

Immediately after the final gas samples had been taken, tap *A* was opened, and the stoppers were removed from the bottles. The tubes were washed with the precipitating reagent, and the tissue was worked up for chemical estimation by one of the methods described in our previous paper [Holmes and Ashford, 1930]. For lactic acid only, trichloroacetic acid was employed, for "free sugar," dilute alcohol, for glycogen, absolute alcohol. Two points only need mention. (*a*) During evaporation of the alcohol, CO_2 escapes and the fluid tends to become strongly alkaline, on account of the breakdown of the bicarbonate to carbonate. To guard against this, a few drops of bromocresol purple were added, and the fluid was kept neutral by the addition of dilute HCl in alcohol, drop by drop as required, during evaporation. (*b*) The calculation of the "extra" lactic acid removed in these experiments was made as follows.

If the initial lactic acid of the tissue = IP, and the amount of lactic acid added = L, and if after oxygenation, for tissue alone, lactic acid remaining = OP and for tissue with lactic addition, lactic acid remaining = OL, then lactic removed from plain sample = $IP - OP$.

Lactic acid removed from sample to which lactic acid had been added = $(L + IP - OL)$.

$$\text{Therefore extra lactic removed} = (L + IP - OL) - (IP - OP)$$
$$= L - OL + OP.$$

Hence the estimation of the initial lactic acid of the tissue was unnecessary, and its omission afforded both a saving of time and tissue. L, OP and OL were estimated chemically.

Table I.

Exp.	Ringer	Initial CO_2 %	p_H Initial	p_H Final	Extra lactic acid removed (mg.)	O_2 consumed (mm.³) Plain	O_2 consumed (mm.³) Lactate	Extra O_2	Lactic equiv. of extra O_2	"Q"	Expected synthesis	Observed synth. (mg.) Free carb.	Observed synth. (mg.) Glycogen	Duration of exp. (min.)	Remarks
14. xi. 30	Bicarb.	5·04	7·20	—	8·54	6070	10670	4600	5·95	1·43	3·6	—	—	240	Oxygen
17. xi. 30	,,	4·83	7·21	—	6·11	5670	7990	2320	2·95	2·1	3·2	—	—	245	Oxygen
21. xi. 30	,,	5·51	7·16	—	10·17	5230	10950	4720	6·37	1·6	3·8	—	—	240	Oxygen
24. xi. 30	Phosph.	0	7·6	—	7·51	4080	5080	1000	1·34	5·6	6·7	—	—	225	Air } Parallel exps.
	,,	0	7·6	—	12·71	4580	8700	4120	5·55	2·3	7·2	—	—	225	Oxygen }
26. xi. 30	Bicarb.	5·03	7·2	—	9·40	6020	8380	2360	3·17	2·9	6·2	—	—	180	Oxygen } Parallel
	Phosph.	0	7·7	—	11·11	4000	6720	2720	3·66	3·0	7·4	—	—	180	Oxygen, Barcroft's } exps.
28. xi. 30	Bicarb.	4·45	7·24	7·29	12·14	4970	8310	3340	4·50	2·7	7·6	0·140	—	180	Oxygen
3. xii. 30	,,	6·54	7·11	—	6·68	5920	8420	2500	3·36	2·0	3·3	0·031	—	180	Oxygen } Parallel exps
	Phosph.	0	7·18	—	8·37	3900	6300	1400	1·88	4·4	6·5	0·120	—	180	Oxygen }
6. xii. 30	Bicarb.	5·38	7·22	7·12	7·74	5270	8720	3450	4·65	1·7	3·1	0·190	—	180	Oxygen
12. xii. 30	,,	5·03	7·14	7·09	—	6860	8990	2130	2·87	—	—	—	−0·22	195	Oxygen. Sodium lactate 1 %
18. xii. 30	,,	6·33	7·15	7·20	5·34	5150	8120	2970	4·00	1·5	1·9	—	+0·12	180	Oxygen. Sodium lactate 0·5 %
15. i. 31	,,	7·45	7·01	—	11·75	—	—	—	—	—	—	—	−0·15	180	Oxygen. Sodium lactate 0·5 %
14. iv. 31	Phosph.	—	7·4	—	11·8	3680	6220	2540	3·41	3·5	8·4	—	—	,,	Oxygen, Barcroft's
17. iv. 31	,,	—	7·4	—	10·0	4100	6425	2325	3·13	3·2	6·8	—	—	,,	Oxygen, Barcroft's
24. iv. 31	,,	—	7·4	—	9·15	4040	6590	2550	3·38	2·7	5·8	—	—	,,	Oxygen, Barcroft's
14. v. 31	,,	—	7·4	—	9·75	4145	6675	2530	3·40	2·9	6·3	—	—	,,	Oxygen, Barcroft's
3. vi. 31	,,	—	7·4	—	10·5	4290	6370	2080	2·80	3·76	7·7	—	—	,,	Oxygen, Barcroft's
13. vi. 31	,,	—	7·4	—	8·14	1460	3710	2250	3·00	2·7	5·1	—	—	,,	Oxygen, Barcroft's. Insulin exp., 1 g. tissue
18. vi. 31*	,,	—	7·4	—	5·5	1510	3000	1490	2·00	2·7	3·5	—	—	135	Oxygen, Barcroft's. Insulin exp., 1 g. tissue

* Initial lactic acid content of tissue = 0·47 mg. per g. Except where otherwise stated, 0·5 % lithium lactate was used.
p_H by indicator method in all experiments with phosphate buffer.

Table I shows the results obtained in a number of experiments. The differences between the initial and final amounts of oxygen were large, and the technique, as a whole, proved satisfactory.

Several important points emerge from inspection of Table I. In the first place, it will be seen that the "quotient" is rather lower (average 2) when the Ringer is buffered by bicarbonate than when it is buffered by phosphate (average 3); the oxygen uptake is distinctly larger both in the presence and

Table II.

Average O_2 uptake of 2 g. of tissue in 3 hours at 37°, with different buffers and gas mixtures

Conditions	Oxygen uptake in mm.³		
	Plain	Lactate	Extra
Air/PO_4	3520	4205	685
Air/CO_2/bicarb.	3570	4420	850
O_2/PO_4	3940	6430	2490
O_2/CO_2/bicarb.	5700	8490	2790

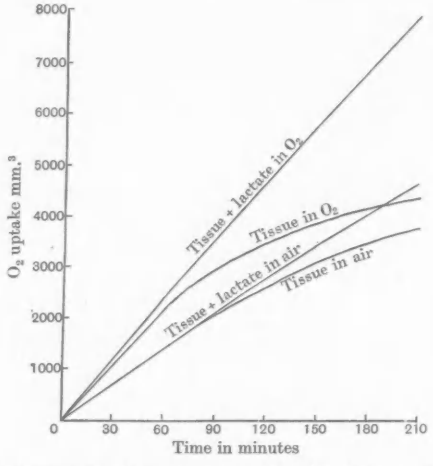


Fig. 2. Effect of oxygen tension on oxygen uptake of brain tissue. All 2 g. tissue. PO_4–Ringer p_H 7·4 at 37°. Lactate 0·5 %.

in the absence of lactate, than when a phosphate buffer is used. Our original experiments were performed in air; and when air is replaced by oxygen, even with phosphate buffer, there is a considerable increase in oxygen consumption (see Fig. 2); nevertheless tissue in bicarbonate buffer and CO_2/O_2 gas mixture shows a still larger uptake than tissue in O_2 and phosphate buffer (see Table II and Fig. 2). In spite of the fall in the quotient in the experiments with O_2/CO_2 and $NaHCO_3$, it will be seen that the net result is that the expected synthesis of carbohydrate is absolutely larger in both sets of experiments carried out in oxygen as compared with those carried out in air. Table II

summarises the differences in oxygen uptake occurring under the different conditions just discussed.

The data given in some cases for "initial" and "final" p_H in Table I, were obtained in order to be sure that, in spite of the large changes in CO_2 percentage which occurred during the experiment (*e.g.* initial 5 %, final 10 %), there was no serious change in p_H.

Table III. *Oxygen uptake of different amounts of chopped brain tissue, in oxygen and Ringer buffered with phosphate* p_H *7·4.*

Amount of tissue taken g.	O_2 uptake per g. in 3 hours mm.³
0·3	2180
0·5	2040
1·0	2025
1·5	2390 (single exp.)
2·0	1970

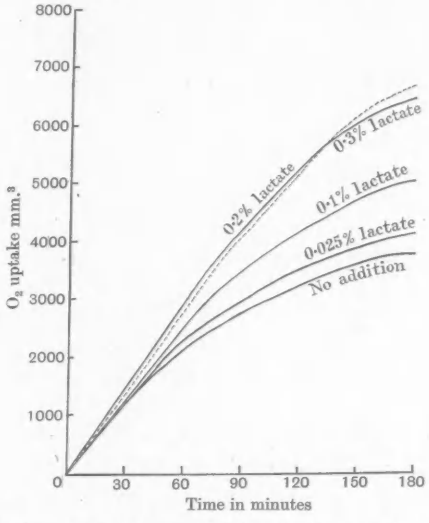


Fig. 3. Effect of varying lactate concentration. 2 g. tissue. p_H 7·4, in O_2 at 37°.

There is no difficulty about determining the initial p_H by means of the quinhydrone electrode. The final p_H was determined in a duplicate flask provided with the necessary electrodes and a device for introducing the quinhydrone at the conclusion of the experiment.

It seems to us that these experiments show that neither the existence of the "quotient" nor the absence of synthesis can be ascribed either to failure of adequate oxygen supply, or to buffering with phosphate, since both phenomena occur equally in air and in oxygen, and in a bicarbonate/CO_2 buffered medium as well as in one buffered with phosphate. Dixon and Elliott [1929]

point out that, if the availability of oxygen is not a limiting factor in experiments with the Barcroft apparatus, then the relationship between oxygen uptake and weight of tissue used will be linear, or, put in another way, the uptake per g. of tissue will be constant, no matter how much tissue is employed in any given experiment. Table III shows that this is the case in our experiments.

One further point should be mentioned here. In most of the earlier experiments, an arbitrary concentration of lithium lactate of 0·5 % was used. Figs. 3 and 4 show the effect of different concentrations of this salt from 0·025 % to 0·8 %. It will be seen that little increase in oxygen uptake is

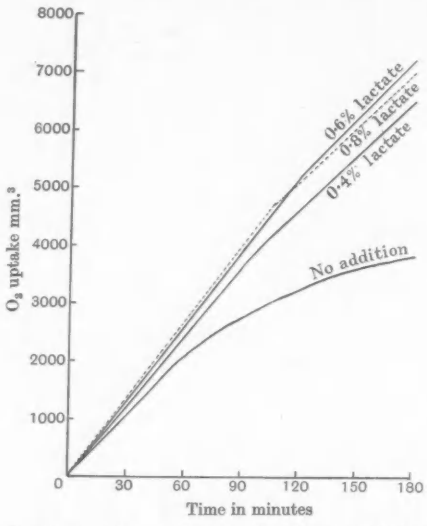


Fig. 4. Varying lactate concentration. 2 g. tissue; p_H 7·4; in O_2 at 37°.

obtained by increasing above 0·4 %. 0·5 % has accordingly been used in all subsequent experiments for convenience of estimation. At lower concentrations, the oxygen uptake curve soon falls off although the initial velocity is in all cases the same. Table IV shows the Meyerhof quotient under different conditions of lactate concentration. It will be seen that at lower concentrations the quotient is somewhat lower, but between 0·3 % and 0·8 % it has an average value of 2·7 to 2·8. These experiments have a further significance in connection with the question of the "sparing" of substrates to be referred to later.

A question of obvious importance is that of the effect of the chopping of brain tissue on its oxidative mechanisms. It has previously been reported by one of us [Holmes, 1930] that the oxygen consumptions of chopped and sliced rabbit brain are about equal. Nevertheless, we wished to try to obtain some

more positive data about the effect of chopping. It was out of the question to use intact mammalian brain (as a control to the chopped tissue), for clearly O_2 could not possibly diffuse quickly enough to supply its needs. On the other hand, with frog's brain in oxygen, and at room temperature, it was to be expected that at the lower temperature the chemical processes of oxidation

Table IV. *Effect of the lactate concentration on the Meyerhof quotient.*

In all experiments 2 g. of tissue were used in O_2 at p_H 7·4 and 37° (Ringer buffered with phosphate).

Concentration of lithium lactate %	Meyerhof quotient
0·025	1·93
0·05	2·26
0·1	1·78
0·2	2·15
0·3	2·54
0·4	2·82
0·5	2·71
0·6	2·96
0·8	2·80

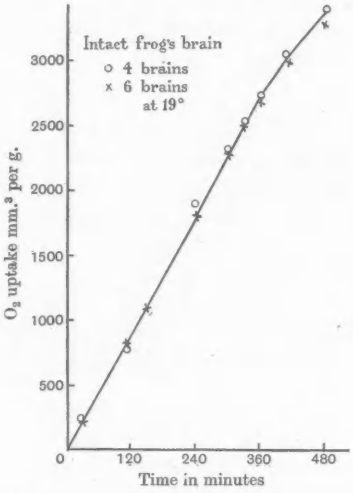


Fig. 5. Oxygen uptake of intact frog's brain at 19°. In O_2 at p_H 7·4.

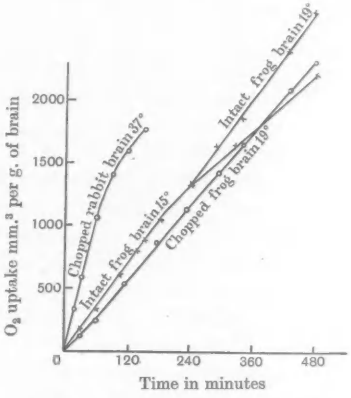


Fig. 6. Oxygen uptake of intact and chopped frog's brain.

would be retarded far more than the physical process of diffusion. We therefore performed some experiments in which the oxygen uptake of intact, freshly dissected frog's brain was determined in the small type of Barcroft apparatus, which is calibrated for 1·0 cc. of fluid. An intact frog's brain is larger than the individual fragments of chopped rabbit's brain used in the other experiments.

Fig. 5 shows the results of two experiments showing that the oxygen uptake per g. is the same when different amounts of tissue are used (4 brains in the one case, 6 in the other).

Fig. 6 shows the effect of chopping on the uptake of frog's brain. The total uptake of the chopped brain is some 16 % less than that of the intact brain, which, in our view, does not suggest that the chopping is likely to have had any very serious deleterious effect upon the oxidative mechanisms. This stands in contrast with the observation of Meyerhof [1919] that chopping of frog's muscle leads to a large increase in the oxygen uptake as compared with the intact muscle.

For purposes of comparison there is added a curve showing the uptake per g. of chopped rabbit brain at 37°.

We think that these experiments tend to show—admittedly, they do not conclusively prove—that our practice of chopping brain tissue is unlikely to interfere with its normal oxidative processes. They do not, of course, throw any light on the possibility of damage to other mechanisms. It must be remembered that less damage is likely to be caused to individual cells in chopping brain, than would be caused by chopping muscle, the long fibres of which would inevitably suffer. It has been shown [Holmes, 1930] that most of the O_2 uptake of brain is due to the cells, and not to the axons.

Section II.

Fate of the lactic acid.

Since the lactic acid which disappeared, over and above that which was accounted for by the "extra" oxygen, was not synthesised to glycogen, or to any form of reducing carbohydrate which could be estimated by the usual methods, we endeavoured, by other means, to trace its fate.

During an informal discussion of our results, it was suggested by Dr B. Woolf, as a possible explanation of them, that all the lactic acid was in reality being oxidised, but that part of it was "sparing" the oxidation of some other substance present in the brain tissue. This would, of course, account for the fact that the "extra" oxygen as measured by the difference between *OL* and *OP* was too small to account for the whole of the lactate which disappeared, simply because other materials were oxidised in *OP* which in *OL* were "spared" and so required no oxygen at all. This suggestion is clearly much to the point; it is also one which it is extremely difficult either to substantiate or to disprove.

It seems clear, however, that it cannot afford the correct explanation, if, in any circumstances, it can be shown that the total lactic acid disappearing would require, if completely oxidised, more oxygen than the total amount of that gas taken up in the manometer containing tissue *plus* added lactate (*OL*). In most of our experiments this is not the case, though, in a good many, there is only a comparatively small margin between the two amounts. If,

however, the experiments are carried out on tissue which has been depleted of its own substrates, either by washing, or by exposure to oxygen, or by the preliminary administration of insulin to the animal, then the oxygen equivalent of the total amount of lactic acid disappearing is very considerably greater than the total O_2 uptake of *OL*. Experiments of this nature are detailed in Table V, and it seems to us therefore unlikely that our results are due simply to the "sparing" of other substrates by lactic acid.

Table V.

Date	O_2 equivalent of total lactic removed mm.³	Total O_2 taken up by *OL* mm.³	Remarks
21. viii. 30	3420	2770	Washed tissue
22. viii. 30	3870	2440	Washed tissue
26. viii. 30	1640	751	Tissue washed and kept 12 hrs. in ice-chest
29. vi. 31	4350	2800	Tissue shaken in O_2 for 3 hrs. before lactate added and readings commenced
1. vii. 31	3185	1564	Tissue shaken in O_2 for 3½ hrs. before lactate added and readings commenced
18. v. 31	4290	3000	Animal rendered hypoglycaemic with insulin before killing
13. v. 31	6360	3710	Animal hypoglycaemic with insulin before killing

If the concentration of lactate is sufficiently low, this relationship does not hold good, at least in the case of washed brain; probably because the enzymes concerned are then no longer saturated with substrate. This point is further discussed at a later stage.

In endeavouring to trace the fate of the lactic acid, it seemed to us to be best to obtain information about the respiratory quotient of the tissue, and particularly about that of the "extra" oxygen. Some preliminary experiments on this point were reported in our first communication [1930]. The technique finally adopted was the one of which the details are given by Dixon and Elliott [1929]. For our purposes, six Barcroft manometers were required, and we have used either the ordinary (3 cc.) or the large type. We may designate them *A*, *B*, *C*, *D*, *E*, *F*. *A* contains tissue and plain Ringer, and the CO_2 is absorbed by sodium hydroxide. *B* is similar to *A*, but contains lactate Ringer. *C* and *E* each contain tissue and plain Ringer, but instead of sodium hydroxide they have a Keilin cup containing saturated oxalic acid solution hung on the sodium hydroxide pot. *D* and *F* are arranged similarly to *C* and *E*, but contain lactate Ringer instead of plain Ringer. All the cups were filled with oxygen.

C and *D* were put into the thermostat and the taps closed as soon as temperature equilibrium was obtained. The acid was immediately spilled into the body of the cup by sharp shaking, and the manometers read until the reading became steady; this took usually 5 to 10 minutes. These readings represented preformed CO_2 in *OL* and *OP* respectively.

The remaining four Barcroft apparatus were then shaken in the thermostat for an appropriate period—2 to 3 hours. The manometers of *A* and *B* were read, and the difference between them gave the "extra" oxygen. At the end of the period, the acid in the cups of *E* and *F* was spilled, and the manometer reading represented the difference between O_2 absorption and total CO_2 in the system at the end of the experiment. Thus, if, for the moment, we take the letters to represent the gas volumes indicated by the readings of the various manometers (*i.e.* the manometer readings × factor)

$A - E = CO_2$ contained in *OP*,
$B - F = CO_2$ contained in *OL*,
$A - E - C = CO_2$ evolved by *OP* (*i.e.* contained in *OP minus* initial CO_2),
$B - F - D = CO_2$ evolved by *OL*.

From the data so obtained, we can calculate the respiratory quotient of the tissue alone, the tissue to which lactate has been added, and the amount of CO_2 corresponding to the "extra" oxygen.

Table VI gives some results obtained by this method. It will be seen that it includes certain experiments performed on the brains of animals with insulin hypoglycaemia. These were performed with the object of discovering whether there was any definite fall in the respiratory quotient as a result of the shortage of lactic acid which has been shown [Holmes and Holmes, 1925] to exist in the brain under these circumstances. No such fall is apparent: evidently, therefore, substrates are still present which maintain the R.Q. at, or close to, unity.

Table VI. *Tables of values found for respiratory quotients.*

Experiments in air				Experiments in O_2			
Date	Tissue only	Extra O_2		Date	Tissue only	Extra O_2	
8. vii. 30	0·935	0·937	Normal animals	8. v. 31	0·924	0·96	Normal animals
11. vii. 30	0·985	0·920	,,	9. v. 31	0·87	—	,,
31. vii. 30	0·758	1·124	,,	12. v. 31	0·87	1·10	,,
5. viii. 30	0·875	1·128	,,	13. v. 31	0·96	0·94	Insulin animals
7. viii. 30	0·909	1·020	,,	18. v. 31	0·89	0·96	,,
20. viii. 30	0·928	0·984	,,				
Mean	0·912	1·018					

The R.Q. of the "extra" O_2 is 1·0 (mean values, 1·018 and 1·03, are certainly within the limits of experimental error) whether the experiments are performed in O_2 or in air. Loebel [1925] gives figures which are somewhat lower than these. This, on the face of it, suggests that the extra oxygen is concerned with the complete oxidation of a corresponding amount of lactic acid. We realise that conclusions from experiments of this kind must be drawn with caution; nevertheless, these findings make it highly improbable that the existence of the Meyerhof quotient depends on a partial oxidation such as the conversion of lactic acid to pyruvic acid, since such a process would give a Meyerhof quotient of 6·0, but R.Q. of the extra oxygen would be 0. The results

obtained in the respiratory quotient experiments could be explained if lactic acid were changed to acetic acid. This would involve the absorption of one molecule of O_2, and the formation of one molecule of CO_2. Apparently, however, this change does not take place, for if it did, either the acetic acid must be further oxidised, or must remain behind unchanged. Brain tissue, however, takes up no extra oxygen with acetate, nor does acetate accelerate the reduction of methylene blue by brain tissue (Thunberg technique); while, again, we were unable to demonstrate an excess of volatile acid in *OL* at the end of the experiment, under conditions in which acetic acid could be quantitatively recovered. Tests for pyruvic acid and acetaldehyde were negative.

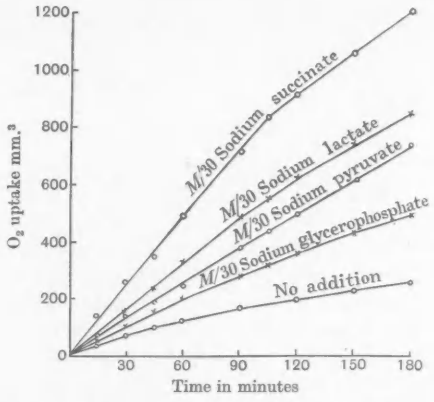


Fig. 7. 0·3 g. tissue in 3 cc. of fluid; p_H 7·4; in air at 37°.

This was again demonstrated by a large scale experiment in which 90 g. of pig's brain were oxygenated, both in the presence and absence of added lactate. The tissues, and also a similar initial sample, were worked up with trichloroacetic acid and filtered. The filtrates were neutralised and concentrated *in vacuo* in the presence of sodium sulphite [*cf.* Neuberg and Kobel, 1930; Case and Cook, 1931]. In no case was a precipitate obtained on adding 2:4-dinitrophenylhydrazine in hydrochloric acid.

In such attempts to follow the course of the disappearance of the lactic acid, the fate of a number of substrates which might possibly act as intermediaries has been investigated, both by the Barcroft aerobic technique and the Thunberg methylene blue reduction technique. Toennissen and Brinkmann [1930] have put forward a scheme involving, for the oxidation of lactic acid by muscle, the formation of pyruvic acid, which in turn gives an unstable 6-carbon compound by the loss of hydrogen. This, in turn, gives succinic and formic acids, which under appropriate conditions may be oxidised. It has already been mentioned that no evidence of pyruvic acid formation has been

obtained in our experiments. In one experiment conducted at room temperature, as opposed to those at 37°, there was very faint and doubtful evidence of the presence of pyruvic acid, as shown by the nitroprusside reaction. In spite of this, we have performed experiments with substrates suggested by Toennissen's work, using equivalent concentrations of each; Fig. 7 shows the oxygen uptake of brain with different substrates. It will be seen that succinate [*cf.* Quastel and Wheatley, 1931], lactate, pyruvate and glycerophosphate are all "oxidised." On the other hand, no evidence of an extra oxygen uptake was obtained with either formate or acetate while mandelate actually caused an inhibition of the order of 50 %. A parallel series of findings is obtained with the methylene blue technique (Table VII).

Table VII. *Effect of various substrates on reduction of methylene blue by brain tissue.*

Hydrogen donators		Not hydrogen donators	
Lactate, Glucose, Succinate, Glycerophosphate	Approx. the same activity	Glycerol, Dihydroxyacetone	No change in reduction time
Pyruvate	Slight change in reduction time	Acetate, Formate, Mandelate	inhibitors

Since our attempts to discover the fate of the lactic acid by qualitative tests for likely substances have given negative results, and since our determinations of the respiratory quotient threw no light on the probable fate of that portion of it which had not been oxidised, we decided, on the advice of Miss M. Stephenson, to perform estimations of total "soluble" carbon. If we estimated the total carbon in protein- and lipoid-free filtrates of *OL* and *OP*, then we should expect that the carbon in *OL* would be equal to that in *OP*, *plus* the carbon added as lactate, *minus* that which had been oxidised to CO_2, and absorbed by the potash during the experiment. If this were not the case, then either the lactate must have been changed to some substance which, though not glycogen, was still not soluble in the precipitating agent used in the preparation of the filtrates, or, alternatively, there must have been some fallacy inherent in our previous experiments or in our reasoning. The method adopted for the estimation of total carbon was the "wet combustion" method of Raistrick [1931]. It was necessary to choose a reagent for precipitating protein and lipoid which was free from carbon; while the presence of halogens in it was undesirable. The acid mercuric sulphate reagent of Hopkins and Cole (10 % mercuric sulphate in 7 % sulphuric acid) was found to be suitable.

2 g. of chopped brain tissue were put into the right-hand cups of each of four large Barcroft manometers, two of which contained plain Ringer, and the other two lactate Ringer. The Ringer in both cases was buffered with phosphate. Each apparatus was filled with oxygen, and shaken for 3 hours in the thermostat, the oxygen uptake being observed as usual. At the end of the experiment the 4 g. of tissue (from the two *OP* Barcroft's) were washed

into a 50 cc. volumetric flask with 10 cc. of the mercuric sulphate reagent, and the necessary amount of water to make up to volume. The 4 g. of *OL* were similarly treated. After standing for not less than 1 hour, the contents of the flasks were transferred to centrifuge-tubes and centrifuged. The supernatant fluid was poured off, and the mercury removed with H_2S. HgS was removed by filtration on a small Büchner funnel, then through an ordinary dry filter-paper, and H_2S by aeration with moist air. 5 cc. of the solution were taken for estimation, and represented one-tenth of the original amount of material.

Table VIII shows the results obtained in these experiments. The figures in the column labelled "calculated" were obtained by adding to the value for *OP* found by estimation the difference between the amount of carbon in the added lactate and that in the CO_2 corresponding to the extra oxygen; in other words, by adding to the value for *OP* the carbon of the "expected synthesis."

Table VIII.

Date	Carbon added as lactate mg.	Carbon equiv. of extra O_2 mg.	Carbon found in *OP* mg.	Carbon calculated in *OL* mg.	Carbon found in *OL* mg.	Percentage difference
25. v. 31	16·16	2·12	13·99	28·03	27·04	3·6
3. vi. 31	16·64	2·24	12·85	27·25	24·65	9·5
14. v. 31	9·91	2·73	12·78	22·69	22·42	1·2
9. vi. 31	16·58	2·78	13·75	27·55	25·89	6·0
					Average	5·07

It will be observed that the values for carbon found in *OL* are less on the average by some 5 % than those "calculated." This may be the result of experimental error; the figures for the calculated value depend upon three separate measurements; (i) the estimation of the added lactate, (ii) the measurement of the "extra" oxygen, and (iii) the estimation of the carbon in *OP*, and some error is likely to creep in. It is, however, possible—since the discrepancy is always in one direction—that it really means that the fate of a small amount of the added lactate is in some way different from that of the remainder.

We feel that these experiments give an assurance that we are dealing with a real phenomenon. We have already stated our reasons for thinking that we are not dealing simply with the sparing of some other substrate by lactic acid. The carbon estimations do not throw any special light on the question of the sparing of substances soluble in mercuric sulphate, as there is clearly no way of identifying the carbon as being derived from lactic acid. But they seem to us to provide some additional evidence against the sparing of protein or of lipoids. If protein were spared by the oxidation of lactic acid, it is surely likely that the molecules would be broken down into smaller and more soluble moieties, and that these would then be oxidised. But in that case, it is unlikely that they would be oxidised so completely as to leave

absolutely no residual soluble carbon, and if any residual carbon were left, it would be estimated in *OP*, and we should find considerable excess of carbon in *OP*. The same argument applies to the sparing of lipoids. We shall return to this point later, when we discuss experiments which deal directly with the question of nitrogen sparing.

We performed a series of experiments to discover whether the missing lactic acid could have been changed into volatile organic acid. Filtrates were prepared, both by precipitation with mercuric sulphate and also with "colloidal iron," using, on several occasions, 16 g. of brain tissue. In the former case, the sulphuric acid was partly neutralised, to avoid risk of charring when the solution became concentrated; in the latter, the solution was acidified with phosphoric acid. The solutions were then distilled *in vacuo*, and the distillate was caught in standard alkali. The hydrochloric acid, some of which (derived from the chlorides of the Ringer) was always present in the distillate, was estimated with silver nitrate, and was deducted from the total volatile acid.

Small amounts of acetic acid were quantitatively recovered in control experiments; nevertheless, in no experiment was there an excess of volatile acid in *OL* over that found in *OP*; though small amounts were present in both samples. Beyond showing, by treatment with mercuric chloride, that this acid was not formic, we have been unable to identify it.

On another occasion, we repeated the experiment, but distilled into bisulphite, instead of into standard NaOH. Traces only of bisulphite-binding substances were present. We have performed experiments in which brain tissue has been shaken in oxygen in the presence of sulphite and lactate. The oxygen uptake is almost entirely inhibited by the sulphite. On applying the nitroprusside test to the tissue and fluid at the end of the experiment, we were unable to satisfy ourselves that pyruvic acid was present. The amount of bound sulphite was in these cases practically zero, and the same in *OL* and *OP*.

Another possibility which suggested itself, was that synthesis of some organic phosphorus compound was taking place which reduced feebly, or not at all, and was therefore not estimated as carbohydrate. We therefore carried out a series of experiments in which we estimated acid-soluble organic phosphorus in the freshly chopped tissue (initial value) and in the tissue which had been shaken in oxygen, both in the presence and absence of added lactate. These experiments were carried out in bicarbonate buffer with O_2/CO_2 atmosphere, to avoid adding phosphates, and estimations of inorganic phosphate, pyrophosphate, and total acid-soluble organic phosphorus (by incineration with sulphuric acid and "perhydrol") were carried out in the trichloroacetic acid filtrates [Briggs, 1924; Lohmann, 1928].

The results of a series of experiments are shown in Table IX. There is consistently slightly more organic acid-soluble phosphorus in *OL* than in *OP*, but *OL* is consistently lower than the initial value. The "expected synthesis" (reckoned as carbohydrate) in all these experiments is large and cannot be

traced to the synthesis of an acid-soluble organic phosphorus compound. Our total carbon experiments make it highly improbable that some insoluble organic phosphorus compound is being formed.

Table IX. *Determinations of acid-soluble phosphorus fractions.*

All figures are mg. P per 2 g. of tissue.

	Initial				Oxygenated without addition (*OP*)				Oxygenated with lactate (*OL*)			
Date	Total	Inorg.	Pyro.	A. s. org.	Total	Inorg.	Pyro.	A. s. org.	Total	Inorg.	Pyro.	A. s. org.
29. ix. 31	1·585	0·965	0·119	0·501	1·85	1·410	0·090	0·350	1·785	1·270	0·155	0·360
30. ix. 31	1·892	1·200	0·031	0·661	1·768	1·270	0·090	0·408	1·745	1·152	0·072	0·521
3. x. 31	1·612	0·907	0·021	0·684	1·870	1·330	0·060	0·480	1·922	1·320	0·044	0·646
9. x. 31	1·594	0·948	0·036	0·609	1·677	1·337	0·023	0·317	0·184	0·356	0·007	0·437
12. x. 31	2·010	1·390	0·160	0·461	2·005	1·470	0·147	0·391	0·960	1·365	0·145	0·445
,,	,,	,,	,,	,,	2·080	1·626	0·140	0·314	0·820	1·294	0·153	0·373
Average	1·738	1·082	0·073	0·583	1·875	1·407	0·091	0·376	1·841	1·293	0·096	0·464

A further significance attaches to these results, in that the consistently slightly greater figure for acid-soluble organic phosphorus in *OL*, than in *OP*, suggests that a certain small amount of sparing of phosphate ester has taken place. The question of "lipoid sparing" will be discussed elsewhere. Loebel [1925] showed that brain was capable to some extent of oxidising hexose-diphosphate, and our results would be in accordance with this finding.

A series of experiments was undertaken, in which total nitrogen and ammonia were estimated in the mercuric sulphate filtrates from the tissue oxygenated both in the presence and in the absence of lactate. Total nitrogen was estimated by that micro-Kjeldahl method in which alcohol is added and the distillation is assisted by aeration, $N/200$ NaOH being used for titration in the presence of de Wesselow's indicator. Ammonia was determined by Watchorn and Holmes's [1927] modification of Stanford's method [1923].

The results given in Table X show that, except for a single experiment, there is always more total nitrogen in *OP* than *OL*, and invariably more ammonia in the former than in the latter.

Table X. *Total soluble nitrogen and ammonia in samples oxygenated without addition* (*OP*) *and in samples oxygenated with added lactate* (*OL*).

All 4 g. of tissue in O_2 at p_H 7·4 for 3 hrs. at 37°.

	Total N (mg.)			Ammonia-N (mg.)		
Date	*OP*	*OL*	Diff.	*OP*	*OL*	Diff.
17. vii. 31	5·35	4·50	0·85	1·054	0·682	0·372
20. vii. 31	6·55	6·60	−0·05	1·256	0·657	0·599
24. vii. 31	6·05	5·29	0·76	1·247	0·772	0·475
30. vii. 31	4·01	3·31	0·70	1·130	0·768	0·362

Loebel [1925] has already shown that less ammonia is formed by brain tissue in oxygen in the presence of glucose or of lactate, and his results are confirmed, with regard to the latter substance, by the evidence which we

present here. The interpretation of these experiments is not at present easy. Clearly, in view of the figures for total carbon, nitrogen which would otherwise be insoluble in mercuric sulphate (*e.g.* protein- or lipoid-nitrogen) cannot have been built with the lactic acid into some nitrogenous compound; but our results would not preclude the possibility of nitrogen already present in some soluble form being transferred to a new compound derived from the lactic acid.

The fact that there is more ammonia in *OP* than in *OL* suggests strongly that the presence of the lactate has inhibited deamination. Pohle [1929] has demonstrated the presence of adenylic acid in brain tissue, and Holmes and Holmes [1925] had earlier stated that pentose was present in brain. At the same time, B. E. Holmes had isolated hypoxanthine, in the form of hypoxanthine silver nitrate, from brain tissue, but as it was subsequently found that Thudichum [1884] had made the same observation at a much earlier date, and as the significance of adenylic acid was not then realised, no mention of this finding was made. In view of this, and of the importance of the deamination of adenine in muscle, it seems very likely that the ammonia is derived, at least partly, from adenine, and that this process is partly inhibited in the presence of lactate. In this case, it is possible that the breakdown of pyrophosphate might also be inhibited. We have, however, found no evidence that this occurs. Table VIII shows that very little pyrophosphate is to be found in the brain under the conditions of our experiments, and that there is no suggestion that its amount is altered by the presence of lactate.

The alternative view—the one which is supported by the change observed in total nitrogen—is that the effect of the lactate is actually to spare protein or lipoid, and that part at least of the N in *OP* represents the deamination of protein or lipoid, or of both; but if such is the case, it must be remembered that the total carbon figures show that the remainder of the molecule must have undergone almost complete combustion.

Loebel [1925] suggests that, if protein is burned, 4–5 molecules of oxygen should be used for every molecule of nitrogen split off. Table IX shows (neglecting the second experiment) that about 0·77 mg. of ammonia-N was apparently "spared" by the lactate. This represents $0{\cdot}77/14 = 0{\cdot}055$ millimoles of ammonia-N, and should, in turn, represent about $0{\cdot}055 \times 4{\cdot}5$ or 0·246 millimoles of oxygen. This oxygen would completely oxidise $\frac{0{\cdot}246}{3} = 0{\cdot}082$ millimoles, or 7·38 mg. of lactic acid.

Lusk [1928] from Loewy's data, states that, in the animal body, 100 g. of (meat) protein would require 138·18 g. of O, and that 8·49 g. of O would be required per g. of urinary N. Thus, 1 mg. of N should correspond to $\frac{138{\cdot}18}{8{\cdot}49} = 16{\cdot}28$ mg. of O and 0·77 mg. would correspond to $\frac{138{\cdot}18}{8{\cdot}49} \times 0{\cdot}77 = 12{\cdot}53$ mg. of O, *i.e.* to $\frac{12{\cdot}53}{32} = 0{\cdot}393$ millimoles of O. This would account for the complete oxidation of $\frac{0{\cdot}393}{3} = 0{\cdot}131$ millimoles, or 11·79 mg. of lactic acid.

Now the average figure which we obtain for the amount of lactic acid

which disappears and is not accounted for by the extra oxygen, is, for 4 g. of brain, 14·68 mg. (mean of 7 experiments in which total carbon is estimated). It will be seen therefore that only about three-fifths of the lactic acid could, on the basis of these figures, have been used for "protein sparing."

If, on the other hand, lipoid (*e.g.* lecithin), instead of protein were spared, and if we assume that the molecular weight of lecithin is 773, then 1 mg. of nitrogen would correspond to 221 mg. of lactic acid. It will be seen, however, from Table IX that the total acid-soluble phosphorus is practically identical in amount both in *OP* and *OL*. It is difficult to picture any manner in which lecithin could be broken up without giving rise to soluble phosphorus compounds, and it seems, therefore, fair to discount the possibility of lipoid sparing having occurred. Table IX shows also that there is only a small liberation of soluble phosphorus in either *OL* or *OP*, the increase being only 0·137 mg. of P in the one case and 0·013 mg. in the other. This is in conformity with the relatively high R.Q. (0·9 and over) which is found even in the absence of lactate. It also falls into line with the experiments of King [1931] who finds that brain tissue does not break down lecithin at all readily.

Table XI.

Date	Lactic acid in *OL* after 1½ hrs. shaking in O_2 mg.	Lactic acid in *OL* after 1½ hrs. in O_2 followed by 1½ hrs. in N_2 mg.	Lactic acid after 1½ hrs. in O_2 followed by addition of glucose, and 1½ hrs. in N_2 mg.	Lactic acid in *OP*, after shaking 1½ hrs. in O_2, followed by glucose and 1½ hrs. in N_2 mg.
4. viii. 31	5·75	5·75	—	—
6. viii. 31	6·40	6·50	14·6	10·05

In the course of experiments still in progress [unpublished], Sherif and Holmes have observed (in confirmation of previous workers McGinty and Gesell [1925], Kinnersley and Peters [1929], and Jungmann and Kimmelstiel [1929]) that if brain tissue is very rapidly cooled in liquid air (in these experiments mice were used), a comparatively low value for lactic acid is obtained, which rises on incubation at 40°. Simultaneous estimations of total carbohydrate show a fall in the latter, coincident in time with the rise in lactic acid, but sometimes less in amount (compare work of Kerly [1931] on muscle). In other words, lactic acid appears from some source other than substances estimated as carbohydrate. It seemed possible that this unindentified lactic acid precursor might, in fact, be the substance into which lactic acid was changed in our own experiments. To test this, samples of brain with added lactate were shaken for 1½ hours in oxygen. One sample was then analysed for lactic acid. The Barcroft apparatus containing another similar sample was then filled with nitrogen, and shaken for a further period of 1½ hours. Analysis showed that this had exactly the same lactic acid content as the sample taken for estimation after 1½ hours in oxygen—no reappearance of lactic acid had taken place under anaerobic conditions. Control experiments showed that,

after 1½ hours' shaking in oxygen, the tissue in nitrogen could still freely form lactic acid from glucose. These results are illustrated by Table XI.

Evidently the lactic acid is not changed into the unknown precursor, which exists, apparently, in the intact brain.

Discussion.

From the experimental evidence before us, we must conclude either that the larger part of the lactic acid which disappears when brain tissue is shaken with lactate in the presence of oxygen is transformed into some substance which has so far eluded identification, or else that all the lactic acid which disappears is in reality oxidised, but in the process "spares" some other substance or substances from oxidation.

The points in favour of the latter explanation are, first, its inherent plausibility. The principle of the "sparing" of one substrate by another in the animal body is well established, and there is no obvious reason why it should not apply to individual isolated organs. Indeed, the occurrence of nitrogen sparing by sugars in the growing kidney has been demonstrated by Watchorn and B. E. Holmes [1927, 1931]. From our own experiments we know that there is actually more soluble nitrogen in brain tissue that has been shaken in oxygen in the absence of lactate than its presence.

Brain tissue certainly contains oxidisable substances other than lactic acid—Thudichum [1884] demonstrated the presence of succinate, and succinate takes up oxygen even more freely than lactate—and probably there are others. The fact that 95 % of the carbon corresponding to the missing lactic acid can be found as soluble carbon is no bar to this explanation, if the line be taken that some soluble, carbon-containing substrate is spared; there is no means of identifying the carbon as derived especially from lactic acid. But it does bear on the question of protein or lipoid sparing, in the sense that it means that, if such sparing occurs, then practically the whole protein or lipoid molecule must be spared. We picture, after all, that hydrolysis must precede oxidation; and if this is so, all the hydrolysed fragments must be completely oxidised—if any were left unoxidised, they would upset the carbon balance. But this complete oxidation seems improbable. Against this explanation too, is the fact that, in tissue depleted of substrate by various methods, more lactic acid disappears than can be accounted for by the oxygen uptake (Table V). Here an explanation on the lines of a "sparing" effect is clearly impossible. We are thrown back on the assumption that some substance is present which, so far, we have failed to identify.

Still further evidence against the "sparing" hypothesis is afforded by a consideration of Figs. 2 and 3 and of Table IV, which shows the effect of varying concentrations of added lactate. We may assume that we have initially present in the tissue unknown substrates, which we may denote by X, and a certain amount of lactic acid l. When shaken in oxygen, suppose these amounts

become X_0 and l_0 (*i.e.* the value given as *OP* in the Tables). In the case of *OL* an amount L of lactic acid was added to the tissue, giving $(X + l + L)$ initially. After shaking in oxygen, suppose the amounts become $x_0 + l_0 + L_0$.

If now $X_0 = x_0$, then the differences in the amounts of oxygen taken up in the two cases should be due to the added lactic acid. If, however, X_0 is not equal to x_0 and is less than x_0, competitive inhibition or "sparing" has taken place. If this inhibition is of a competitive nature [Haldane, 1930], then an increase of L should cause a diminution in the amount of X oxidised, *i.e.* x_0 should be greater than X_0. In effect more of the oxygen taken up will have been used in the oxidation of the lactic acid than is shown by the difference of the two oxygen uptakes, *i.e.* more lactic acid is disappearing but the amount of "extra oxygen" remains the same. Hence the Meyerhof quotient should increase as we increase the concentration of the added lactate. On the other hand, assuming that, in actual fact, total oxidation of lactic acid takes place as the R.Q. suggests, the ratio

$$\frac{\text{O}_2 \text{ equivalent of total lactic acid disappearing}}{\text{Total oxygen uptake}}$$

should approach unity or fall as the lactic concentration is increased. It should be noted that 2 g. of the chopped tissue would contain about 2·7 mg. of lactic acid, and that this amount is almost maximal, *i.e.* aerobic glycolysis does not take place (as it does in muscle) except to a very small extent, owing to the absence of precursor in the tissue [Holmes and Holmes, 1925]; Ashford

Table XII. *Ratio of* $\frac{\textit{Oxygen equivalent of total lactic acid removed}}{\textit{Total oxygen uptake}}$ *at different initial concentrations of added lactate.*

Approx. conc. Li lactate %	Initial lactic acid g.	Final lactic acid g.	Lactic acid removed g.	O_2 equiv. of lactic acid removed mm.³	Total O_2 uptake mm.³	Ratio
0·025	3·7	0·56	3·14	2,350	4710	0·5
0·05	4·7	0·68	4·02	3,000	4875	0·615
0·1	6·9	1·6	5·31	4,450	5000	0·89
0·2	11·97	2·885	9·1	6,750	6450	1·05
0·3	17·0	4·65	12·35	9,200	6700	1·37
0·4	18·4	7·31	11·1	8,300	6480	1·28
0·5	22·4	10·32	11·1	8,260	6300	1·31
0·6	26·7	13·2	13·5	10,000	7110	1·40
0·8	34·2	20·1	14·1	10,500	7000	1·44

and Holmes, 1929]. At the lowest concentrations employed therefore, the amount of added lactic acid is small, or comparable with the amount already in the tissue. The findings have already been noted, *viz.* that at the lowest concentrations the Meyerhof quotient is smaller but that between 0·3 % and 0·8 % there is little change. This makes it unlikely that competition between substrates is taking place, or in other words, probably no "sparing" occurs. Further evidence of this is given in Table XII. It will be seen that the ratio

$$\frac{\text{O}_2 \text{ equivalent of total lactic acid disappearing}}{\text{Total oxygen uptake}}$$

is less than unity at low concentrations, but afterwards increases above unity, whereas if the suggestion made above were correct, it should decrease with increased concentration of lactate.

Summary.

1. Experiments are described in which it is shown that a Meyerhof quotient can be obtained if brain tissue is shaken with lactate in the presence of a bicarbonate buffer, in an O_2/CO_2 atmosphere. A technique is described by which the use of manometric methods is avoided. In spite of the existence of the "quotient," no carbohydrate synthesis occurs.

2. The O_2 uptake is greater in the presence of a CO_2-bicarbonate buffer than in the presence of phosphate and increases with increased oxygen tension in both cases.

3. There is little difference between the oxygen uptake of intact and chopped frog's brain, measured in O_2 at room temperature, in the presence of phosphate buffer.

4. The respiratory quotients, both of the brain tissue alone and of the "extra oxygen," are close to unity, even in the case of animals rendered hypoglycaemic by insulin injection.

5. With brain tissue, glucose, lactate, succinate and glycerophosphate all act as hydrogen donators to methylene blue. Glycerol and dihydroxyacetone are without effect on the reduction time; formate, acetate and mandelate prolong it.

6. Estimations of total carbon in protein-free filtrates of tissue shaken with and without lactate indicate that the "extra oxygen" gives a true measure of the lactic acid oxidised.

7. There is no evidence of formation of volatile acid, or of any substance capable of binding bisulphite, from lactic acid in the presence of brain tissue.

8. Inorganic, pyro-, and acid-soluble organic phosphorus have been estimated. There is evidence of a small increase both in inorganic and in total phosphorus, at the expense of organic phosphorus, both in the presence and in the absence of lactate; but there is no evidence of the synthesis of any phosphorus compound from the fraction of the lactic acid which disappears and is not accounted for by oxidation.

9. There is more non-protein nitrogen, and more ammonia, in filtrates from brain tissues which have been aerated in the absence of lactic acid, than in its presence.

10. The possibility of accounting for the disappearance of lactate on the lines of a "sparing" effect on some other substrate is discussed, and it is shown that such an explanation is probably not the correct one.

Our thanks are due to Sir F. G. Hopkins, for his interest in this work.

REFERENCES.

Ashford and Holmes (1929). *Biochem. J.* **23**, 748.
Briggs (1924). *J. Biol. Chem.* **59**, 255.
Case and Cook (1931). *Biochem. J.* **25**, 1319.
Dixon and Elliott (1929). *Biochem. J.* **23**, 812.
Haldane, J. S. (1920). Methods of air analysis. (Griffin.)
Haldane, J. B. S. (1930). Enzymes, p. 7. (Longmans, London.)
Holmes (1930). *Biochem. J.* **24**, 914.
—— and Ashford (1930). *Biochem. J.* **24**, 1119.
—— and Holmes (1925). *Biochem. J.* **19**, 496.
Jungmann and Kimmelstiel (1929). *Biochem. Z.* **212**, 347.
Kerly (1931). *Biochem. J.* **25**, 671.
Kinnersley and Peters (1929). *Biochem. J.* **23**, 1126.
King (1931). *Biochem. J.* **25**, 799.
Loebel (1925). *Biochem. Z.* **161**, 219.
Lohmann (1928). *Biochem. Z.* **202**, 466.
Lusk (1928). The science of nutrition. (Saunders, London.)
Meyerhof (1919). *Pflüger's Arch.* **175**, 20.
McGinty and Gesell (1925). *Amer. J. Physiol.* **75**, 70.
Neuberg and Kobel (1930). *Ber. deutsch. chem. Ges.* **63**, 1039.
Pohle (1929). *Z. physiol. Chem.* **185**, 281.
Quastel and Wheatley (1931). *Biochem. J.* **25**, 117.
Raistrick (1931). *Phil. Trans. Roy. Soc. Lond.* B **220**.
Stanford (1923). *Biochem. J.* **17**, 847.
Thudichum (1884). The chemical constitution of the brain. (Baillière, Tindall and Cox, London.)
Toennissen and Brinkmann (1930). *Z. physiol. Chem.* **187**, 137.
Warburg (1926). Ueber die Stoffwechsel der Tumoren, p. 198. (Springer, Berlin.)
Watchorn and B. E. Holmes (1927). *Biochem. J.* **21**, 1391.
—— —— (1931). *Biochem. J.* **25**, 843.

CCXXII. THE DISTRIBUTION OF THE VITAMIN B COMPLEX.

III. FRUITS.

By MARGARET HONORA ROSCOE[1].

From the Division of Nutrition, Lister Institute, London.

(*Received October 31st, 1931.*)

Most of the previous work on the distribution of vitamin B in fruits was carried out before the differentiation of the complex into the antineuritic vitamin B_1 and the antidermatitis vitamin B_2, and as a rule the results of the older experiments give only rough indications of the presence of both constituents of the complex. These have been summarised by Sherman and Smith [1931].

In the work described in the present paper, apple, banana, orange and tomato, the fruits in most common use in this country, were tested for their vitamin B_1 and vitamin B_2 content respectively. Vitamin B_4 [Reader, 1929; 1930, 1, 2] has not been tested for independently. As it was not being supplied in the basal diet used for the vitamin B_1 tests, these indicated the content of vitamin B_1 and B_4 combined.

This work is a continuation of that on the vitamin B_1 and B_2 content of foodstuffs previously carried out in this laboratory [Aykroyd and Roscoe, 1929; Aykroyd, 1930; Chick and Copping, 1930; Roscoe, 1930, 1931]. A comparison is given in this paper of all the results hitherto obtained.

Methods.

The methods employed were those described in the previous papers [Roscoe, 1930, 1931], in which the growth of young rats is used as criterion.

In the vitamin B_1 estimations the diets contained unpurified caseinogen and the vitamin B_2 was supplied as autoclaved yeast extract (F5A). For the vitamin B_2 estimations purified caseinogen was used and vitamin B_1 was provided as Peters's antineuritic concentrate from yeast (YE) [Peters, 1924; Kinnersley and Peters, 1925].

The fruits were prepared by peeling, coring or removing the pips, and then mincing. The water content of the pulps so obtained was reduced by exposing to the air in thin layers at 37°. The fruits were bought from time to time throughout the experiments. No particular variety was adhered to.

The reduced pulps were incorporated in the diets in varying percentages (reckoning from the dry weights), but the rats were only allowed to consume

[1] Beit Memorial Fellow.

enough of these diets to provide a given amount of the fruit each day, the remainder of their requirements being satisfied with a vitamin-free diet.

THE VITAMIN B_1 CONTENT OF CERTAIN FRUITS (Table I).

Table I. *Vitamin B_1 content of certain fruits as demonstrated by weight increase of rats during 5 weeks on diets containing various percentages of the fruit pulps.*

(Vitamin B_2 supplied as autoclaved yeast extract (F5A).)

Material tested	Amount eaten daily: Wet weight g.	Amount eaten daily: Dry weight g.	No. of rats	Increase in weight in 5 weeks (averages, g.)
Orange	2·5	0·25	4	50
	5·0	0·5	1	85
Tomato	6·25	0·5	4	38
	12·5	1·0	4	82
Banana	2·3	0·5	2	No growth
	4·6	1·0	4	51
Apple	5·5	1·0	4	32
	11·0	2·0	4	66

The amounts referred to in the following paragraph are the dry weights consumed daily.

Orange proved a rich source of the antineuritic vitamin; rats eating as little as 0·25 g. gained 50 g. in weight in 5 weeks, those eating 0·5 g., 85 g. *Tomato* was about half as good as orange; rats receiving 0·5 g. gained 38 g., those with 1·0 g., 82 g. *Banana* was one-quarter as rich as orange; with 1·0 g. the increase in weight was only 51 g. *Apple* was even poorer; with as much as 2·0 g. the increase in weight in 5 weeks was only 66 g.

In Table II the results obtained with these fruits, together with the values for the other foodstuffs previously published, are tabulated in the order of their vitamin B_1 potency. The amounts shown are the dry weights needed by

Table II. *The vitamin B_1 content of various foodstuffs (dry weights).*

Material	Daily dose needed to promote increase in wt. of 50–60 g. in 5 weeks g. dry wt.		Potency (yeast = 100)
Yeast (brewer's)	0·05	<0·25	100
Wheat germ	0·1		50
Liver (ox)	<0·5	0·25–0·5	10–20
Watercress	<0·3		
Lettuce	0·3		
Orange	0·25		
Etiolated cabbage	0·3 –0·5		
Carrot	0·25–0·5		
Spinach	<0·5		
Green cabbage	<0·95	0·5–1·0	5–10
Turnip	0·5–1·0		
Tomato	0·5–1·0		
Egg-yolk	0·5–1·0		
Onion	0·5–1·0		
Meat (ox muscle)	1·0–1·5	1·0–2·0	2·5–5·0
Banana	1·0		
Potato	1·0–2·0		
Apple	1·0–2·0		

young rats, when given as sole source of vitamin B_1, to produce an increase in weight of 50–60 g. in 5 weeks. These values have been used to calculate the vitamin B_1 potency in terms of that of yeast. Yeast being taken as 100, the materials are placed in groups with potencies of over 20, 10–20, 5–10 and 2·5–5, respectively.

It will be seen that the orange is as good a source of vitamin B_1 as any of the substances tested, except yeast and wheat germ. Its potency lies between 10 and 20 and is equal to those of the green leaf vegetables, liver and carrot. Tomato has a potency of between 5 and 10, equal to those of turnip, egg-yolk and onion. Banana and apple have potencies between 2·5 and 5, equal to those of meat and potato.

The vitamin B_2 content of certain fruits (Table III).

Orange, *tomato* and *banana* all proved to be equal sources of this vitamin. In each case rats receiving 1·0 g. daily increased in weight by about 30 g. in 5 weeks and those receiving 2·0 g. by about 60–70 g. *Apple* was again the

Table III. *The vitamin B_2 content of certain fruits as demonstrated by weight increase of rats during 5 weeks on diets containing various percentages of the fruit pulps.*

(Vitamin B_1 supplied as antineuritic yeast concentrate (YE).)

Material tested	Amount eaten daily: Wet weight g.	Amount eaten daily: Dry weight g.	No. of rats	Increase in weight in 5 weeks (averages, g.)
Orange	10·0	1·0	4	29
	20·0	2·0	5	69
Tomato	12·5	1·0	2	No growth
	25·0	2·0	4	67
Banana	4·6	1·0	4	35
	9·2	2·0	4	62
	18·4	4·0	3	102
Apple	5·5	1·0	2	34
	11·0	2·0	4	47
	22·0	4·0	4	66

poorest of the fruits tested; rats receiving 1·0 g. increased in weight 34 g. in 5 weeks but 4·0 g. were needed to produce 66 g. increase.

In Table IV these fruits are placed in order of their vitamin B_2 contents, together with the other foodstuffs previously tested. The amounts needed to promote an increase in weight of 50–60 g. in 5 weeks are given and from these have been calculated their potencies when yeast is taken as 100.

The fruits tested do not stand very high as regards their vitamin B_2 content; it was lower than the vitamin B_1 content. Orange, tomato and banana are equal to wheat germ and onion, poorer than the green leaf vegetables, root vegetables, meat and milk, but richer than the cereals, pulses or potatoes. Apple has a content equal to that of the cereals.

In Table V are shown the vitamin B_1 and vitamin B_2 potencies, respectively, of the various foodstuffs, when the fresh weights needed to promote

Table IV. *The vitamin B_2 content of various foodstuffs (dry weights).*

Material	Daily dose needed to promote increase in wt. of 50–60 g. in 5 weeks g. dry wt.		Potency (yeast = 100)
Yeast (brewer's)	0·1	< 0·25	100
Liver (ox)	0·12		80
Egg-white	0·3–0·6	0·25–0·5	20–40
Watercress	0·4		
Green cabbage	0·4		
Spinach	0·4		
Milk (cow's)	0·7	0·5–1·0	10–20
Meat (ox muscle)	0·5–0·75		
Lettuce	0·6		
Etiolated cabbage	0·4–0·8		
Carrot	0·5–1·0		
Turnip	1·0		
Egg-yolk	1·0		
Wheat germ	1·0–2·0	1·0–2·0	5–10
Onion	1·6		
Orange	1·0–2·0		
Banana	1·0–2·0		
Tomato	< 2·0		
Peas (dried)	2·0–3·0	2·0–4·0	2·5–5
Wheat (whole)	2·0–3·0		
Apple	2·0–4·0		
Potato	2·0–4·0		
Maize (whole, white)	2·0–3·5		
Millet	> 4·0	> 4·0	< 2·5
Maize (whole, yellow)	> 4·0		
Rice	> 4·0		

Table V. *The vitamin B_1 and B_2 potencies of various fresh foodstuffs.*

The potencies have been calculated by comparing the amounts of the fresh materials needed to promote 50–60 g. growth in young rats during 5 weeks, with the amount of fresh yeast needed (*i.e.* as source of vitamin B_1, 0·05 g., as source of vitamin B_2, 0·1 g.).

Potency	Vitamin B_1	Vitamin B_2
More than 100	Wheat germ	Liver (ox)
100	Yeast (brewer's)	Yeast (brewer's)
20–100	Liver (ox)	Wheat germ
	Egg-yolk	Egg-yolk
10–20		Meat (ox muscle)
		Egg-white
		Watercress
		Peas (dried)
		Wheat (whole)
		Spinach
		Maize (whole, white)
		Green cabbage
5–10	Meat (ox muscle)	Milk
	Watercress	Lettuce
	Orange	Etiolated cabbage
	Lettuce	Carrot
	Spinach	Banana
	Etiolated cabbage	
	Carrot	
	Banana	
2·5–5	Potato	Potato
	Green cabbage	Apple
	Tomato	Orange
	Turnip	Tomato
	Apple	Turnip
Less than 2·5	Onion	Onion

50–60 g. growth in 5 weeks are compared with the amount of fresh yeast needed.

It is noticeable that with a few exceptions the materials tested do not vary widely in their content of vitamins B_1 and B_2 respectively. Yeast, wheat germ, liver and egg-yolk have potencies of both vitamins greater than 20, but all the remaining foods lie between 2·5 and 20, with the exception of onion which is lower.

A general similarity is also exhibited in the distribution among the foodstuffs of the two vitamins, the materials when placed in order of value forming two very similar series (see Table V). There are however marked exceptions. Liver and meat have a considerably higher proportion of vitamin B_2 than of B_1 (vitamin B_1 potency/vitamin B_2 potency = 1/4), and the green leaf vegetables, egg-yolk, banana and turnip a slightly higher proportion (1/2), that is, these take higher places in the vitamin B_2 series than in the vitamin B_1 series. On the other hand, wheat germ has a much higher proportion of vitamin B_1 than of B_2 (8/1), and orange and the whole cereals a slightly higher (2/1); these, therefore, take higher places in the vitamin B_1 series.

Egg-white is unique in that it is the only known natural source of vitamin B_2 without vitamin B_1. No natural source of vitamin B_1 without vitamin B_2 is known.

These results may be compared with those of previous investigators. Apples have always been found to be poor sources of "vitamin B" [Weatherby and Waterman, 1928]. Oranges were here found to be richer in the antineuritic vitamin, than had been thought, about 4 cc. of juice, not 10 cc. [Sherman, 1926], being sufficient for normal growth when vitamin B_2 was being adequately supplied; 20 cc., not 10 cc. [Willimott, 1928], were needed, however when the juice was the sole source of vitamin B_2. Tomatoes, as previously shown [House, Nelson and Haber, 1929], were here found to be rather poorer than oranges in vitamin B_1. Bananas were tested for their vitamin B_1 and B_2 contents separately by Eddy [1927]. The present assays do not agree with his. This may be due to the fact that Eddy based his estimations on weight maintenance for both vitamins, whereas the weight of rats can be maintained without vitamin B_2 if vitamin B_1 is supplied. On this basis the vitamin B_2 potency was found by him to be considerably higher than the vitamin B_1 potency, whereas in this work it was lower. In Eddy's work also 6 g. of fresh banana when given as source of vitamin B_2 were found to support nearly normal growth, here more than 17 g. were needed.

Summary.

1. The vitamin B_1 content of orange was the highest of the fruits tested, being one-fifth that of yeast (dry weights) and equal to that of the green leaf vegetables or liver. The value of tomato was rather less than one-tenth that of yeast, banana one-twentieth and apple still lower.

2. The vitamin B_2 content of the fruits tested was lower. The value of

orange, tomato and banana was rather less than one-tenth and of apple about one-twentieth that of yeast (dry weights).

3. The vitamin B_1 and B_2 potencies of all the foodstuffs tested in this laboratory have been calculated taking yeast as standard. These are tabulated in order of magnitude, firstly comparing dry weights and secondly fresh weights.

4. A large number of the foodstuffs tested had the same proportion of vitamin B_1 to vitamin B_2 as had yeast. Liver, meat, the green leaf vegetables and egg-yolk were richer in vitamin B_2 than in vitamin B_1, while the reverse was true of wheat germ, orange and the whole cereals.

My thanks are due to Dr H. Chick for advice and criticism throughout the course of this work, and to Miss D. Graetz for help with the experimental animals.

REFERENCES.

Aykroyd (1930). *Biochem. J.* **24**, 1479.
—— and Roscoe (1929). *Biochem. J.* **23**, 483.
Chick and Copping (1930). *Biochem. J.* **24**, 1764.
Eddy (1927). *Proc. Soc. Exp. Biol. Med.* **25**, 125.
House, Nelson and Haber (1929). *J. Biol. Chem.* **81**, 495.
Kinnersley and Peters (1925). *Biochem. J.* **19**, 820.
Peters (1924). *Biochem. J.* **18**, 858.
Reader (1929). *Biochem. J.* **23**, 689.
—— (1930, 1). *Biochem. J.* **24**, 77.
—— (1930, 2). *Biochem. J.* **24**, 1827.
Roscoe (1930). *Biochem. J.* **24**, 1754.
—— (1931). *Biochem. J.* **25**, 1205.
Sherman (1926). Chemistry of food and nutrition, 3rd edition, p. 406. (New York.)
—— and Smith (1931). The vitamins, 2nd edition. (New York.)
Weatherby and Waterman (1928). *J. Ind. Eng. Chem.* **20**, 968.
Willimott (1928). *Biochem. J.* **22**, 67.

CCXXIII. THE EFFECTS OF COPROPHAGY IN RATS DEPRIVED OF THE VITAMIN B COMPLEX.

By MARGARET HONORA ROSCOE[1].

From the Division of Nutrition, Lister Institute, London.

(*Received October 31st, 1931.*)

Introduction.

The presence of "vitamin B" in the excreta of birds and mammals has long been established. The antineuritic vitamin was found by Cooper [1914] in alcoholic extracts of faeces from hens and rabbits receiving normal diets, and Portier and Randoin [1920] found it in the faeces, but not in the urine, of rabbits which had been kept on diets deficient in vitamin B. Its presence was attributed by these authors either to unabsorbed residues from the food or to synthesis by bacteria in the intestines.

Muckenfuss [1918], Gaglio [1919] and Van der Walle [1922] all found the antineuritic vitamin in adsorbates from the urine of animals fed on normal diets, but it was reduced in starving animals and absent after 3 weeks' deprivation of "vitamin B."

Later it was observed that animals on diets completely or partially deficient in "vitamin B" frequently consumed their own faeces, the result being, when suboptimal doses of the vitamin were being supplied, a greatly improved rate of growth. Thus Steenbock, Sell and Nelson [1923] observed that such rats grew normally with half as much "vitamin B" in the diet as was needed by the non-coprophagous rats. They considered that an additional supply of "vitamin B" was being obtained from the unabsorbed residue of the food, since in absence of "vitamin B" from the diet coprophagy did not prolong life.

Dutcher and Francis [1923], however, kept coprophagous rats on diets completely deficient in "vitamin B" for 5–7 months. They considered this prolongation of life to be due to the re-ingestion of excreted stores of "vitamin B," an explanation supported by the failure of rats to benefit by coprophagy if it was not started till after the first few weeks of vitamin depletion, when the stores of the vitamin were exhausted [Steenbock, Sell and Jones, 1923].

The limited capacity of animals for storing "vitamin B" made it improbable that their stores, even when wastage through the faeces was prevented, could be sufficient to protect against symptoms of "vitamin B" deficiency during the long periods of observation. It was suggested that only minute

[1] Beit Memorial Fellow.

quantities of the vitamin were used at a time, or that none was actually destroyed, the action being catalytic, but against these theories there is the certainty of loss in the portion of the faeces which is not consumed, and the probability of loss in the urine. The theory was therefore advanced that "vitamin B" was synthesised by bacteria in the alimentary canal of coprophagous rats and evacuated with the faeces, from which it was assimilated. This was the more likely as a number of bacteria have been shown to synthesise the vitamin B complex even when grown on synthetic media free of any traces of the vitamin [Schieblich, 1929, 1930, 1, 2].

In support of this theory Heller, McElroy and Garlock [1925] found a marked increase in spore-bearing organisms, which were able to synthesise "vitamin B," in the faeces of coprophagous rats. These had completely disappeared, however, after 3 weeks.

Bechdel *et al.* [1928] found that cows could be reared to maturity on "vitamin B"-deficient diets. Their rumens were shown to contain the vitamin, as the result, it was thought, of synthesis by a small unknown bacterium which was isolated. Coprophagy did not seem to be necessary in order that the vitamin complex should be obtained from the bacteria or their products.

In none of these researches was the vitamin B complex differentiated into its components. When rats were used the experiments dealt therefore with the whole complex; when the tests were based on the prevention or cure of polyneuritis in birds the vitamin B_1 was alone being considered.

Kennedy and Palmer [1928] showed that both vitamins B_1 and B_2 were supplied in the faeces and considered that a third factor was also present supplementing the "vitamin B"-deficient diet; this appears, however, to have been due to an inadequate supply of vitamin B_2.

Scheunert, Schieblich and Rodenkirchen [1929] studied the effect of coprophagy in rats receiving diets containing raw starch, but the greater number of their rats became refected [Fridericia *et al.*, 1927; Roscoe, 1927]. These workers did not apparently realise the difference between their results and those obtained by other workers on coprophagy in whose experiments refection had been prevented by using dextrin in the diet instead of starch [Roscoe, 1927].

Subsidiary reasons, which have been put forward to account for the improved condition of coprophagous animals, are (i) that the faeces act as roughage which is thought to prolong the existence of "vitamin B"-deficient animals [Heller, McElroy and Garlock, 1925], and (ii) that these rats do not suffer from the same disabling discomfort as those in which coprophagy is prevented by the use of cages with open wire floors [McCollum, Simmonds and Becker, 1925].

In this paper experiments are described involving an accurate investigation of coprophagy in rats deprived of the entire vitamin B complex, of vitamin B_1 alone or of vitamin B_2 alone. An attempt was also made to determine the origin of the B-vitamins which these rats acquired through eating their faeces, and to see whether this was similar to that of the vitamin B obtained by refected rats.

Methods.

The rats were kept in the cages used in this laboratory for all work on the B-vitamins. These are made of perforated zinc with floors of galvanised wire netting of $\frac{1}{3}$ inch mesh. The floors of the cages, through which the faeces fall freely, are raised 2″ above the peat bedding. In some of the experiments the rats were housed in glass jars 6″ in diameter, on raised floors made from the same wire netting. Both the coprophagous rats and their controls were kept in these cages; no results could therefore be attributed to differing degrees of comfort enjoyed by the rats.

The peat was covered with a fine wire gauze which allowed the passage of the urine but not of the faeces, from this floor the faeces were collected daily. They were dried overnight at 37°, ground, in some cases weighed, and fed to the rats on the following day. In this form they were more readily consumed than when fresh.

The rats were fed on a basal diet complete except for B-vitamins. Vitamins A and D were supplied as a daily dose of cod-liver oil. The diet consisted of caseinogen 20 parts, rice starch 60, salt mixture 5, cotton-seed or arachis oil 15 parts. This diet was usually steamed for 3–4 hours before using so that the starch was rendered completely soluble.

When vitamins B_1 and B_2 were given these were provided as Peters's antineuritic concentrate from yeast (YE) [Peters, 1924; Kinnersley and Peters, 1925] and as an autoclaved yeast extract (F5A) [Roscoe, 1930], respectively.

No distinction has been made in this work between vitamins B_1 and B_4 [Reader, 1929, 1930, 1, 2] but it was known that the yeast concentrate used as source of vitamin B_1 having been made with acid alcohol contained vitamin B_4 [Reader, 1929]. The yeast extract used as source of vitamin B_2, having been autoclaved for 5 hours, would not contain any vitamin B_4. The rats were, therefore, either deficient in vitamins B_1 and B_4, or deficient only in vitamin B_2.

Young rats were weaned at the age of 3 weeks and received the basal diet and cod-liver oil as soon as they weighed 40 g. Litters 1,000, 2,000 and 4,000 then received their supplementary doses of faeces. Litters 45,000, 51,000 and 52,000 all received their doses of faeces and of vitamins B_1 or B_2 after 1 week on the basal diet alone.

Experimental.

Exp. 1. Growth and prolongation of life in rats deprived of the entire vitamin B complex.

Litters as in Table I.

In Table I are collected the data relating to coprophagous rats and their non-coprophagous litter-mates on a diet deficient in the whole vitamin B complex.

The results with rats receiving an uncooked diet will be considered under Exp. 5.

The coprophagous rats on the cooked diets all remained alive for much longer than their controls. Three developed neuritis after 9½, 9½ and 13 weeks respectively and four after 21, 22, 35 and 36 weeks. The rest were in good health when the experiments were terminated after 8 weeks (3 rats), 19 weeks (2 rats) and 9, 10, 15 and 16½ weeks respectively.

For the greater part of the period of observation the rats did not grow

Table I. *Life histories of coprophagous rats and their non-coprophagous litter-mates on diets deficient in the entire vitamin B complex.*

(In brackets are given the average weekly weight increase.)

Rat No.	Coprophagous	Rat No.	Non-coprophagous
Uncooked diet:			
1,005 ♂	Lost weight for 4 weeks. Died	1,009 ♂	Became refected after 1 week
1,004 ♂	Became refected after 1 week	1,010 ♀	Lost weight for 3 weeks. Dosed
1,006 ♂	,, ,, 2 weeks		
2,008 ♂	Lost weight for 4 weeks. Died	2,006 ♂	Lost weight for 3 weeks. Died
2,009 ♀	,, 3 ,, Dosed	2,007 ♀	,, 4 ,, ,,
		2,010 ♂	,, 3 ,, Dosed
Cooked diets:			
1,001 ♂	Slow growth for 8 weeks (1 g.). Experiment stopped	1,007 ♂	Neuritis after 6 weeks
1,002 ♂	,, 8 ,, (3 g.). ,,	1,008 ♂	Lost weight for 4 weeks. Dosed
1,003 ♀	,, 8 ,, (2 g.). ,,		
2,001 ♂	*Grew (5 g.) for 16 weeks with one break of 4 weeks.* Lost weight. Neuritis after 22 weeks	2,002 ♀	Lost weight for 3 weeks. Died
2,011 ♂	Slow growth for 8 weeks (1 g.). *Grew for 9 weeks (6 g.).* Stationary then lost weight. Neuritis after 21 weeks	2,003 ♂	,, 5 ,, Dosed
2,004 ♀	*Grew (5·5 g.) for 16 weeks with one break of 3 weeks.* Stationary then lost weight. Neuritis after 35 weeks		
2,005 ♂	4 weeks (0 g.). *Grew (7 g.) for 13 weeks with one break of 3 weeks.* Stationary then lost weight. Neuritis after 36 weeks.		
4,004 ♀	Weight stationary for 9 weeks. Neuritis after 9½ weeks	4,001 ♂	Lost weight for 5 weeks. Died
*4,006 ♂	,, ,, 9 ,, ,, 9½ ,,		
4,002 ♂	Grew for 8 weeks (3 g.). Stationary for 5 weeks. Neuritis after 13 weeks		
*4,005 ♂	Grew for 11 weeks (2 g.). *Then for 8 weeks (6 g.).* Experiment stopped		
4,003 ♀	,, 11 ,, (1·5 g.). *Then for 8 weeks (8 g.).* ,,		
*4,007 ♀	,, 11 ,, (2 g.). *Then for 5½ weeks (6 g.)* ,,		
51,384 ♂	,, 10 ,, (1 g.) Experiment stopped	51,383 ♂	Lost weight for 5 weeks. Neuritis
52,390 ♂	,, 6 ,, (2 g.). Lost weight for 3 weeks. Experiment stopped	52,389 ♀	,, 5 ,, Dosed
52,391 ♀	,, 6 ,, (2 g.). *Then for 9 weeks (5 g.).* Experiment stopped		

* These rats received faeces which had been sterilised.
The litter of the rats is indicated by the figures in the fourth and fifth places.

appreciably (average 2 g. per week) (see Fig. 1, curve *C* ii), but in 8 out of the total 16 there was a sudden increase in the rate of growth after varying periods of coprophagy (Table I, italics and Fig. 1, curve *C* iii). This growth was not very rapid (average 6 g. per week) but it was steady and continued till the animals weighed about 100–110 g., but never beyond this. Four of the rats were killed while still growing (rats 4,003, 4,005 and 4,007), two others stopped growing, lost weight and died of neuritis (rats 2,001 and 2,011) and the other two remained stationary in weight for several months and then lost weight and died of neuritis (rats 2,004 and 2,005).

No difference is apparent in the improvements due to coprophagy, between those rats which consumed their faeces from the start (litters 1,000, 2,000 and 4,000) and those which received a vitamin-free diet for 1 week first (litters 51,000 and 52,000).

From this experiment it appeared that coprophagous rats on cooked diets deprived of the entire vitamin B complex acquired, through the eating of their faeces, sufficient of the complex to prolong their lives for considerable periods. In all the cases investigated death finally occurred as a result of neuritis due to vitamin B_1 deficiency.

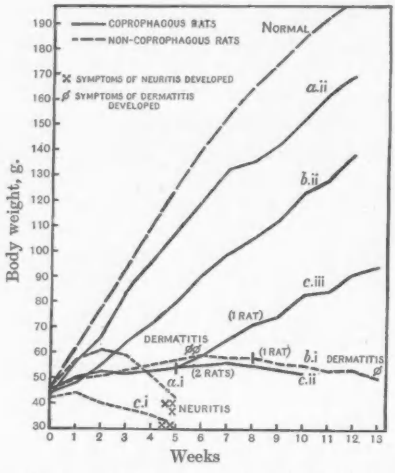


Fig. 1. *Exp.* 3. The growth of coprophagous rats on diets: (*a*) deprived of vitamin B_1, (*b*) deprived of vitamin B_2, (*c*) deprived of the entire vitamin B complex, compared with that of non-coprophagous controls on the same diets, and with normally growing rats.

The curves are in each case, until the vertical lines, averages for 3 rats, 2 males and 1 female.

Exp. 2. The quantities of faeces excreted and consumed by rats deprived of the vitamin B complex.

In these experiments (litters 2,000 and 4,000) two series of rats received a diet devoid of the vitamin B complex. Records were kept of the dry weights of faeces excreted and fed and of the amounts which they failed to eat (see Table II). The percentages of faeces consumed varied from 67 to 77 % in one litter, from 90 to 100 % in the other, the average consumption for any week never fell below 44 %. The growth, expressed as weekly weight increase, is not apparently related to the amount of faeces eaten during the whole time of the experiment.

In Table II are also shown the amounts of faeces eaten during two successive periods in the histories of 7 of the rats, in the first of which they were losing or stationary and in the second gaining in weight. For 3 rats where consumption was low in the first period (57–69 %) it was slightly raised in the second (71–73 %). For the others consumption was high in the first period (77–100 %) and was not increased in the second. It cannot therefore

be said that the growth was due to an increase in the amount of faeces consumed, but the figures do perhaps indicate that growth was only possible when more than about 70 % of the faeces excreted were being eaten.

After the first few weeks of coprophagy the dry weight of the faeces excreted became very high, rising to as much as 1·6 and 1·8 g. daily, whereas rats receiving the same diet adequately supplemented with B-vitamins, but in which coprophagy was prevented, excreted only 0·4–0·5 g. daily. This increase in amount is probably the result of the accumulation of undigestible products of metabolism which are continually re-ingested.

Table II. *Growth of rats and percentage of faeces consumed on a diet deficient in the vitamin B complex when coprophagy was allowed.*

The figures are given for the whole experiment, for several weeks when weight was being lost or was stationary, and for the immediately ensuing weeks when weight was increasing.

	Whole experiment			Period of loss of weight			Period of gain of weight		
Rat No.	Weeks	Av. weekly growth g.	% of faeces eaten	Weeks	Av. weekly growth g.	% of faeces eaten	Weeks	Av. weekly growth g.	% of faeces eaten
2,001 ♂	14	3·3	77	4	−2·5	77	4	4·5	74
2,004 ♀	16	4·0	71	3	−2·7	59	3	6·0	71
2,005 ♂	16	3·9	67	3	−3·3	69	3	9·3	72
2,011 ♂	16	3·7	69	5	−1·0	57	5	5·2	73
	Average	3·7	71						
4,002 ♂	13	1·8	93	—	—	—	—	—	—
4,003 ♀	19	4·2	90	3	−1·0	95	3	10·1	90
4,004 ♀	9	0·6	97	—	—	—	—	—	—
4,005 ♂	19	4·0	98	3	1·7	100	3	6·6	96
4,006 ♂	9	0·0	100	—	—	—	—	—	—
4,007 ♀	19	2·0	98	5	0·4	100	5	5·2	93
	Average	2·1	96						

Exp. 3. The effect of coprophagy in rats deprived of vitamins B_1 and B_2 respectively.

In this experiment (litters 45,000, 51,000, 52,000 and 66,000) the effect of coprophagy in rats deprived of either of vitamin B_1 (series (*a*)) or of vitamin B_2 (series (*b*)) only was investigated.

The experiment lasted 10 weeks. Table III shows the increase in weight during the first 5 and second 5 weeks respectively and the average weekly growth over the whole period for these rats and for their non-coprophagous litter-mates. Growth figures for rats from the same litters, deprived of the whole vitamin B complex (series (*c*)) are shown for comparison. In Fig. 1 are shown the average growth curves of these rats.

Series (*a*). The weight increase of the coprophagous rats receiving vitamin B_2 in their diet (as F5A) but deprived of vitamin B_1, approached the normal, being 64 g. (average) in the first 5 weeks, falling to 46 g. (average) in the second 5 weeks. Control litter-mates, prevented from coprophagy, grew for 2 weeks then lost weight and all developed neuritis by the 5th week (see Fig. 1, curves *a* i and *a* ii). The weight increase during the second 5 weeks

gave the more accurate measure of the vitamin B_1 obtained from the faeces owing to the exhaustion of reserve stores of vitamin B_1, which were sufficient to allow the growth of the controls for 2 weeks. In a further experiment with rats receiving a diet containing vitamin B_2, in which coprophagy did not start till the body weights were stationary, weight increase during the first 5 weeks of coprophagy was 55 g. (average).

These experiments indicate that enough vitamin B_1 was obtained from the faeces to prevent neuritis and support fairly good growth.

Series (*b*). The coprophagous rats receiving vitamin B_1 in their diet (as YE) but no vitamin B_2 increased in weight during the first 5 weeks by 34 g. (average) during the second 5 weeks by 41 g. (average). The control rats prevented from coprophagy, grew an average of 12 g. in the first 5 weeks, by

Table III. *Exp. 3. Growth of coprophagous rats and their non-coprophagous controls on diets* (*a*) *deprived of vitamin* B_1, (*b*) *deprived of vitamin* B_2, (*c*) *deprived of the entire vitamin B complex.*

Coprophagous rats				Non-coprophagous rats		
	Weight increase (g.)				Weight increase (g.)	
Rat No.	1st 5 weeks	2nd 5 weeks	Average weekly	Rat No.	1st 5 weeks	
(*a*) Diet complete except for vitamin B_1:						
45,338 ♂	68	51	12	45,333 ♂	+11, −15 in 32 days.	Neuritis
45,339 ♂	68	56	12	45,334 ♂	+15, −16 in 35 days.	Neuritis
45,335 ♀	57	30	9	45,340 ♀	+19, −22 in 35 days.	Neuritis
Average	64	46	11		+15, −18	
(*b*) Diet complete except for vitamin B_2:						
45,336 ♂	25	50	7·5	45,332 ♂	13	Dermatitis in 42 days
51,385 ♂	34	24	6	51,378 ♂	13	Dermatitis in 42 days
45,337 ♀	40	46	9	—	—	
52,392 ♀	39	46	8·5	52,386 ♀	11	Dermatitis in 98 days
Average	34·5	41·5	8		12	
(*c*) Diet complete except for the vitamin B complex:						
51,384 ♂	5	4	1	51,383 ♂	+2, −12 in 33 days.	Neuritis
52,390 ♂	12	−5	1	52,388 ♂	+2, − 4	
52,391 ♀	11	(29)	2 (6)	52,389 ♀	+2, −18 in 30 days.	Neuritis
Average	9	0	1		+2, −11	

the 6th week 2 had developed dermatitis, the weight of the other remained stationary and it developed dermatitis after 14 weeks (see Fig. 1, curves *b* i and ii).

Thus when vitamin B_1 was being supplied in the diet an amount of vitamin B_2 sufficient to prevent dermatitis and allow subnormal growth appears to have been obtained by coprophagy.

Series (*c*). These rats, which were deprived of the entire vitamin B complex, increased in weight by only 9 g. (average) in the first 5 weeks of the experiment. This increase was much less than that observed in either series (*a*) or (*b*). During the next 5 weeks 2 rats continued to show no significant increase in weight though the other started to grow (see Fig. 1, curves *c* i, ii and iii).

Thus when no vitamin B_1-containing yeast concentrate (YE) or vitamin

B_2-containing yeast extract (F5A) was being supplied in the diet, less of each vitamin was obtained through coprophagy.

It was possible that the increased production of vitamins B_1 and B_2 respectively in the rats of series (*a*) and (*b*) was due to some factor common to the materials supplying vitamins B_2 (F5A) and B_1 (YE) respectively, or alternatively, the vitamin B_1 obtained from the faeces might have been increased when vitamin B_2 was present in the diet and the vitamin B_2 when vitamin B_1 was in the diet. To test these theories a further experiment was carried out.

Coprophagous rats (litter 66,000) were given the vitamin B_1 yeast concentrate (YE) autoclaved under the same conditions as the vitamin B_2 yeast extract (F5A) (5 hours at 120°, p_H 4·7). The concentrate now contained no vitamin B_1. These rats, however, grew as well as those receiving the unheated concentrate, 47 g. (average) weight increase in 5 weeks. It was not therefore vitamin B_1 that was stimulating the production of vitamin B_2 and it seems probable that there was some heat-stable factor present in both the preparations from yeast (YE and F5A) which stimulated the production of both vitamins B_1 and B_2 in coprophagous rats.

To summarise Exps. 1–3, the faeces of rats deprived of the vitamin B complex appeared to contain small amounts of vitamins B_1 and B_2. These amounts were in some cases increased so that growth of from 6 to 8 g. per week was allowed, either spontaneously, after varying periods of coprophagy (curve *c* iii, Fig. 1), or when some factor present in autoclaved yeast extracts, but not either vitamin B_1 or B_2, was being supplied in the diet (curves *a* ii and *b* ii, Fig. 1).

Exp. 4. The effect of feeding sterilised faeces to rats.

If the vitamin B complex obtained by coprophagous rats were present in the faeces as the result of synthesis by intestinal flora, the rôle of coprophagy might be explained in three ways: (1) the vitamin B complex might be acquired from the faeces, not having been accessible at the place of synthesis, (2) the faeces might re-infect the gut with the synthesising organism, or (3) they might contain some substance which stimulates the growth in the intestines of the synthesising organism. The effect of feeding sterilised faeces was investigated to test the second hypothesis. A group of rats was fed on faeces sterilised by heating in a moist state for 3 hours at 80° before drying. This degree of heating, which was not enough to destroy the B-vitamins, was found to kill the bacterial flora, cultures only very occasionally showing any growth.

The histories of these rats are shown in Table I, litter 4000. No difference was detected between the rats receiving sterilised faeces (marked with an asterisk) and their controls receiving faeces dried at 37° without previous treatment. The beneficial effect of coprophagy in rats deprived of the vitamin B complex is not therefore due to a constant re-infection of the gut with a vitamin B-producing organism.

Exp. 5. A comparison of the effects of coprophagy in rats on diets containing insoluble starch (uncooked) and on diets containing soluble starch (cooked).

In Table I are given the histories of 5 coprophagous rats on uncooked diets. Three of these rats thrived no better than their non-coprophagous littermates, death occurring as a result of vitamin B_1 deficiency after about 4 weeks. The other 2 rats, together with one of the controls, became refected.

This result is a great contrast to that obtained with rats which were fed with a cooked diet containing soluble starch, and which were always benefited to some extent by coprophagy. It should be noted again that the American workers on coprophagy all used dextrin as their carbohydrate and so avoided refection.

The condition of refection is obscure but it has been shown to depend on coprophagy. It can be transmitted from an affected rat to another by feeding the faeces, and it can only continue if the rats eat their own faeces. Fridericia and his co-workers concluded that micro-organisms in the faeces of refected rats were responsible for the transmission of refection and probably also for its continuance, for transmission was prevented when the faeces were heated for 10 minutes at 100°. Schieblich and Rodenkirchen [1929, 1, 2] have indeed isolated a *Vibrio*-like organism from the faeces and gut of refected rats, which they think to be the responsible agent.

The state of refection has many points in common with that of the coprophagous rats described in this paper. In both cases rats receiving no B-vitamins in the diet remained in health for considerable periods and even exhibited growth. This was much more marked in the refected rats, however. In both cases the state was induced and maintained in rats by the eating of their own faeces, though refected animals only ate a small quantity, the others a large proportion (see Exp. 2).

The two phenomena are however different. Refection only occurred in rats receiving diets containing raw starch and ceased if the diet were cooked, whereas the prolongation of life and the growth observed in these experiments only occurred in rats receiving diets in which the starch had been rendered soluble by cooking.

It is however possible that the organism supposed to be responsible for refection might be also functioning in the coprophagous non-refected rats, though unable to synthesise as much vitamin B complex when the diet was cooked. An experiment was therefore carried out in which the cooked diet of 5 growing coprophagous rats was changed to an uncooked diet.

These were rats 45,338 and 45,335 receiving vitamin B_2 but no vitamin B_1, rats 45,336 and 45,337 receiving vitamin B_1 but no vitamin B_2, and rat 52,391 receiving no vitamin B complex. After the first week on the new diet all lost weight steadily, their condition became worse and refection did not develop.

The same organism did not therefore appear to be responsible for the possible synthesis of the B-vitamins in the two conditions. If synthesis by

micro-organisms in the gut is responsible for the supply of the vitamins to refected rats and to the unrefected coprophagous rats respectively, it appears that those causing refection flourish in a gut filled with raw starch, and the others in one containing soluble starch.

Discussion.

The following conclusions may be drawn from these experiments as to the origin of the vitamin B complex which enables coprophagous rats on diets deficient in the complex, but containing soluble starch, to live and thrive.

It is improbable that the B-vitamins were obtained from the body stores excreted in the faeces, for firstly, there was wastage of the vitamins in the unconsumed faeces and even when faeces passed during the 1st week of vitamin B depletion were discarded, there was no lessening of the good effect of coprophagy (a result in contradiction to that obtained by Dutcher and Francis [1923]). Secondly, it frequently happened that whereas at the start of an experiment coprophagous rats only maintained their weights, after several weeks, when their body stores would be depleted, they started to grow, without any increase in consumption of faeces.

Synthesis of the vitamin B complex in these animals is therefore indicated, and this theory is supported by the fact that the amounts of the B-vitamins available for the coprophagous animal appeared to be increased by the presence in the diet of a factor, contained in autoclaved yeast extracts, but not one of the known B-vitamins (Exp. 3). This is difficult to explain except on the assumption that synthesis was being stimulated by this factor.

It seems probable that the synthesis was carried out by bacteria. This was not proved however, and an experiment designed to show whether the benefit of coprophagy was due to reinfection of the gut with bacteria gave negative results. Also it is difficult to explain by this theory the very steady and consistent growth obtained in the two series of animals (*a*) and (*b*) in Exp. 3, which were deficient in vitamins B_1 and B_2 respectively. For it is hard to suppose that the micro-organisms present in different animals would always produce the same amounts of these vitamins, or that they would be eliminated in the same amounts.

The exact rôle of coprophagy is not apparent. It may be that the vitamins were synthesised by bacteria in the intestines, but that they were not liberated here but only when the bacteria were digested higher up in the gut after re-ingestion of the faeces, or the faeces may contain some factor which stimulates the growth of bacteria.

No connection could be traced in these experiments between the process whereby these coprophagous rats, receiving diets containing soluble starch, obtained their B-vitamins and that whereby refected rats obtained their supplies. If both processes are based on a bacterial synthesis, it appears that different bacteria are involved in each.

SUMMARY.

1. A quantitative method of controlling the degree of coprophagy in rats, by collecting, drying and feeding the faeces is described.

2. The life of rats receiving diets deficient in the vitamin B complex or its constituents and containing soluble starch was prolonged considerably when they ate their faeces, and in some cases growth also took place. This was not prevented by a preliminary week of depletion during which coprophagy was not permitted.

3. From 44 to 100 % of their faeces was consumed by the individual rats. Growth did not occur when less than 70 % was being consumed, but it was not necessarily proportional to increased consumption.

4. The above facts are most easily explained by the theory that in coprophagous rats there is a synthesis of the B-vitamins, probably by bacteria in the lumen of the gut.

5. This synthesis was stimulated by the presence in the diet of a heat-stable factor, contained in autoclaved yeast extracts and differing from the recognised B-vitamins.

6. The rôle played by the re-ingested faeces is not evident. The efficacy of sterilised faeces showed that the action was not due to the re-infection of the gut with a synthesising organism. It may be that the B-vitamins are obtained directly from the faeces, or the faeces may contain a substance which stimulates the growth of synthesising bacteria already established in the gut.

7. When the rats were fed on uncooked diets containing raw starch, coprophagy did not benefit them unless refection occurred. Changing from a cooked to an uncooked diet resulted in the development of symptoms of deficiency of the B-vitamins.

8. Refection is a condition distinct from that observed in coprophagous rats fed on diets containing soluble starch. It only occurred in rats fed on diets containing raw starch. No connection between the two conditions could be traced.

My thanks are due to Dr H. Chick for advice and criticism throughout the course of this work and to Miss D. Graetz for help with the care of some of the experimental animals.

REFERENCES.

Bechdel, Honeywell, Dutcher and Knutsen (1928). *J. Biol. Chem.* **80**, 231.
Cooper (1914). *J. Hygiene*, **14**, 12.
Dutcher and Francis (1923). *Proc. Soc. Exp. Biol. Med.* **21**, 189.
Fridericia, Freudenthal, Gudjonnsson, Johansen and Schonbye (1927). *J. Hygiene*, **27**, 70.
Gaglio (1919). *Il Policlinico*, **26**, 1381.
Heller, McElroy and Garlock (1925). *J. Biol. Chem.* **65**, 255.
Kennedy and Palmer (1928). *J. Biol. Chem.* **76**, 607.
Kinnersley and Peters (1925). *Biochem. J.* **19**, 820.

McCollum, Simmonds and Becker (1925). *J. Biol. Chem.* **63**, 547.
Muckenfuss (1918). *J. Amer. Chem. Soc.* **40**, 1606.
Peters (1924). *Biochem. J.* **18**, 858.
Portier and Randoin (1920). *Compt. Rend. Acad. Sci.* **170**, 478.
Reader (1929). *Biochem. J.* **23**, 689.
—— (1930, 1). *Biochem. J.* **24**, 77.
—— (1930, 2). *Biochem. J.* **24**, 1827.
Roscoe (1927). *J. Hygiene*, **27**, 103.
—— (1930). *Biochem. J.* **24**, 1754.
Scheunert, Schieblich and Rodenkirchen (1929). *Biochem. Z.* **213**, 226.
Schieblich (1929). *Biochem. Z.* **207**, 458.
—— (1930, 1). *Biochem. Z.* **220**, 394.
—— (1930, 2). *Biochem. Z.* **225**, 212.
—— and Rodenkirchen (1929, 1). *Biochem. Z.* **213**, 234.
—— —— (1929, 2). *Biochem. Z.* **213**, 245.
Steenbock, Sell and Jones (1923). *J. Biol. Chem.* **55**, 411.
—— —— and Nelson (1923). *J. Biol. Chem.* **55**, 399.
Walle, Van der (1922). *Biochem. J.* **16**, 713.

CCXXIV. OXIDATION-REDUCTION POTENTIALS OF *STAPHYLOCOCCUS* CULTURES.

II. EFFECT OF BACTERIOPHAGE.

By LESLIE FRANK HEWITT.

From the Belmont Laboratories (London County Council), Sutton, Surrey.

(*Received October 31st, 1931.*)

In the presence of the appropriate bacteriophage the fall in potential of *B. dysenteriae* (Shiga) cultures is completely inhibited whilst in the case of *B. coli* [Hewitt, 1931, 1] the fall in potential is merely delayed. These effects on the oxidation-reduction potential follow the effect on the proliferation of the bacteria, since growth is almost completely inhibited by bacteriophage in the case of *B. dysenteriae* (Shiga) but is merely delayed in the case of *B. coli*. It was concluded therefore that the effect of bacteriophage is merely to inhibit or delay the fall in potential associated with the growth and metabolic activities of the bacteria.

The oxidation-reduction potentials observed in *Staphylococcus aureus* cultures have already been described [Hewitt, 1930] and this paper describes the effect of bacteriophage on the potentials of the cultures.

General methods.

The general methods and apparatus used were those described previously [Hewitt, 1931, 2]: 0·1 cc. of a 24-hour broth culture of *S. aureus* was inoculated into 8 cc. of medium, and the potentials were measured periodically in each experiment.

The sample of bacteriophage and sensitive strain of *S. aureus* used were kindly provided by Dr C. H. Andrewes. Before use the bacteriophage was "subcultured" several times by adding it to 6-hour broth cultures of *S. aureus*, incubating for a further 18 hours and then filtering through Seitz filters. In each experiment 0·25 cc. of phage filtrate was added to each 8 cc. of culture.

Results.

The oxidation-reduction potential curves of the cultures without bacteriophage were substantially the same as those described previously [Hewitt, 1930] with a different strain of *S. aureus*. The effect is described below of the addition of bacteriophage to the cultures both at the time of inoculation with the organisms and after 5 hours' incubation.

Stationary aerobic cultures (Fig. 1). The potential during the first 6 hours' incubation fell as rapidly in the presence of bacteriophage as in its absence, but after this, whereas the potential in the culture without bacteriophage continued to fall and reached the level E_h − 0·18 v., in the phage culture the potential rose slightly and did not at any time fall below the level − 0·10 v. It will be seen later that these effects, similar in kind but exaggerated in degree, are observed also in the case of aerated cultures. When bacteriophage

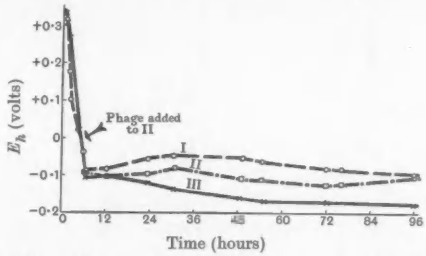


Fig. 1. Stationary aerobic broth cultures of *Staphylococcus aureus*. I. Phage added at beginning of incubation. II. Phage added after 5 hours' incubation. III. No phage added.

was added to a *Staphylococcus* culture after 5 hours' incubation the potential of the culture continued to fall for a time but then ceased to fall, and the lowest level of potential reached (− 0·12 v.) was intermediate between that of the culture without bacteriophage and that of the culture to which bacteriophage had been added at the beginning of incubation. At the end of the first 12 hours' incubation marked growth was visible in the culture not containing bacteriophage; no growth was visible in the culture to which bacteriophage had been added at the beginning of incubation, and there was very faint growth in the culture to which bacteriophage had been added after 6 hours' incubation. In the culture to which bacteriophage had been added at the beginning of incubation a faint trace of growth was observed first after 30 hours' incubation and it will be seen in Fig. 1 that after this period a slight fall in potential occurred. The initial fall in potential had occurred without marked proliferation of the organisms. A similar phenomenon was observed in the case of *B. coli* but not with *B. dysenteriae* (Shiga) [Hewitt, 1931, 1]. It was observed that cultures to which bacteriophage had been added were more acid than those not containing bacteriophage after 6 days' incubation. This will be commented upon later.

Aerated cultures (Fig. 2). It was found in aerated cultures, as in stationary aerobic cultures, that the presence of bacteriophage had no appreciable effect on the potential during the first 5 hours' incubation, but after this period the potential began to rise rapidly in the cultures containing bacteriophage whilst in the cultures without bacteriophage the potential continued to fall slowly. After 24 hours' incubation the potential in the phage culture had fallen again and at this period growth was first visible. In the culture not containing

bacteriophage growth was observed after only 6 hours' incubation. In the culture to which bacteriophage was added after 5 hours' incubation the potential continued to fall for a time but then rose rapidly and later fell again. This second fall in potential was accompanied by proliferation of the bacteria. The hump-shaped potential-time curves appear to be characteristic of the aerated phage cultures, and are associated with changes in the proliferation of the organisms.

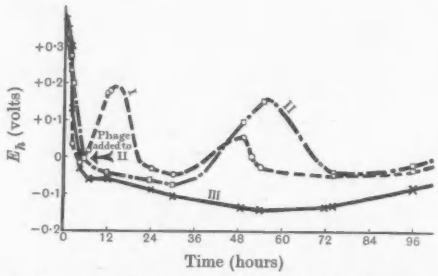


Fig. 2. Aerated broth cultures of *S. aureus*. I. Phage added at beginning of incubation. II. Phage added after 5 hours' incubation. III. No phage added.

Discussion.

The effects of bacteriophage on the oxidation-reduction potentials of *S. aureus* are entirely different from the effects observed with *B. dysenteriae* (Shiga) but are similar in some respects to those observed with *B. coli* [Hewitt, 1931, 1]. In the case of the Shiga bacillus proliferation of the organisms and the associated fall in potential are almost completely inhibited in the presence of bacteriophage, but in the case of *B. coli* proliferation and the fall in potential are merely delayed. On the whole the effect of bacteriophage on the potentials of cultures follows the effect on the growth of the organisms. An exception to this correlation between growth and the fall in potential occurs in the case of stationary aerobic 1 % glucose broth cultures of *B. coli* containing bacteriophage in which the potential shows an initial fall despite absence of bacterial growth. In the case of *S. aureus* also there is an initial fall in potential of cultures in the presence of bacteriophage without appreciable proliferation of the bacteria both in aerobic and aerated plain broth. In aerated cultures containing bacteriophage the potential rises rapidly after this initial fall but later it falls again when visible growth becomes apparent in the culture. The initial fall in potential appears to be due to the enzymic activities of non-proliferating bacteria, but the subsequent fall occurs only when marked growth of the culture takes place. This succession of alterations in potential, namely a fall, rise and fall, gives the potential-time curves of aerated cultures a typical hump-shaped appearance. In stationary aerobic cultures the potential-time curves follow a similar course to those of aerated cultures

except that the rise in potential after the initial fall is much less marked since the oxygen supply is more restricted.

There is a fall in potential during the lysis of *M. lysodeikticus* by lysozyme [Hewitt, 1931, 1] and it was tentatively suggested that this fall in potential might be due to the activity of the enzymes of the lysing bacteria. The fact that in cultures of *S. aureus* containing bacteriophage an initial fall in potential is observed without visible growth of the bacteria suggests that the enzymes of non-proliferating bacteria may be producing this fall in potential and lends some support to the hypothesis that the fall in potential during lysis of *M. lysodeikticus* is a manifestation of the enzymic activity of the lysing bacteria.

After several days' incubation *Staphylococcus* cultures containing bacteriophage are frequently more acid than the cultures not containing bacteriophage. This observation may be accounted for by the fact that during the initial phases of growth bacterial cultures in general become acid owing to production of fatty acids *etc.*, but in later stages of growth of some organisms the reaction becomes more alkaline owing to the oxidation of the acids to carbonates. The more acid reaction of many cultures containing bacteriophage may be due to normal occurrence of the acid phase of metabolism and inhibition by the bacteriophage of the subsequent alkaline phase. This hypothesis is supported by the observation that in *S. aureus* cultures the initial fall in potential occurs in the presence of bacteriophage but the subsequent course of the potential-time curve is altered when bacteriophage is present.

From experiments on *B. coli* Young [1929] concludes that oxidations may be effected by organisms which are dead as judged by their reproductive power.

Summary.

1. The potential of *S. aureus* cultures falls initially even in the presence of bacteriophage despite the delay in the proliferation of the bacteria.

2. In cultures containing bacteriophage the potential soon ceases to fall and commences to rise, but a further fall in potential occurs at the time when growth first becomes apparent in the cultures.

3. Apparently the second fall in potential, but not the initial fall, is dependent on the proliferation of the bacteria.

The author is indebted to Dr C. H. Andrewes for the culture and bacteriophage and to Dr R. G. White and Dr E. W. Todd for their encouraging interest.

REFERENCES.

Hewitt (1930). *Biochem. J.* **24**, 676.

—— (1931, 1). *Biochem. J.* **25**, 1447, 1452, 1641.

—— (1931, 2). Oxidation-reduction potentials in bacteriology and biochemistry. (London County Council.)

Young (1929). *Biochem. J.* **23**, 831.

CCXXV. SYNTHESIS AND CRYSTAL SPACINGS OF CERTAIN LONG-CHAIN PARAFFINS, KETONES AND SECONDARY ALCOHOLS.

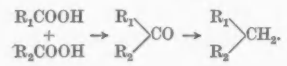
By STEPHEN HARVEY PIPER, ALBERT CHARLES CHIBNALL, SYDNEY JOHN HOPKINS, ALFRED POLLARD, JAMES ANDREW BUCHAN SMITH and ERNEST FRANK WILLIAMS.

From the Wills Physical Laboratory, University of Bristol, and the Biochemical Department, Imperial College of Science and Technology, South Kensington.

(*Received October 31st, 1931.*)

Introduction.

It has long been known that plant waxes contain many substances, notably paraffins and long-chain primary alcohols, besides true waxes such as ceryl cerotate. Attention has been drawn recently to the possible path of metabolism in the plant of some of these substances by Channon and Chibnall [1929] who found that the wax fraction of cabbage leaves (which they refer to as the crude hydrocarbon) consisted chiefly of *n*-nonacosane and 15-nonacosanone. It was suggested by them that paraffins and ketones of this type might arise from the lower fatty acids by condensation and reduction in the following way:



Unfortunately, in the case of the cabbage ketone and paraffin, this postulates the presence of pentadecoic acid at some stage in the synthesis, and as acids containing odd numbers of carbon atoms have not yet been satisfactorily identified in nature, it was felt that the question of the immediate precursor of the ketone should be left over until further experimental evidence was available.

During the past two years we have been investigating the composition of the ethereal extract of numerous forage grasses and, as these all contain a high proportion of wax material, an opportunity has thus been afforded of obtaining more of the required evidence.

We were encouraged to undertake this work by the success attained by Francis, Piper and Malkin [1930] in their researches into the constitution of the long-chain fatty acids occurring in natural products. These authors obtained their standardising data from synthetic fatty acids of 14–26 carbon

atoms, and from mixtures of these. They concluded that the identity of these fatty acids could only be established with certainty when the M.P. and 001 spacings of two crystal modifications were known. They were able to show quite definitely that the naturally occurring acids, usually referred to as arachidic, lignoceric, cerotic and montanic acids, were all mixtures of *n*-fatty acids. Furthermore, the crystal spacings obtained enabled them in certain favourable cases to deduce the probable composition of the mixture.

In the case of the plant waxes the X-ray method of analysis has again proved strikingly successful and the constitution of the main components, *e.g.* paraffins, ketones, primary alcohols, secondary alcohols and fatty acids, has been suggested with such certainty that confirmation by appropriate synthesis has been relatively easy. To attain this result we have had not only to perfect the methods of separating the various components of the waxes, but also to prepare highly pure synthetic products, especially paraffins, in order to have accurate standards of comparison. The present paper, which deals with these synthetic products and their X-ray analysis, is therefore a necessary forerunner to further papers dealing with the components of the various plant waxes.

EXPERIMENTAL.

Preparation of fatty acids.

(The M.P.'s in brackets refer to Francis, Piper and Malkin [1930].)

Kahlbaum's *myristic* and *palmitic* acids were recrystallised four times from large volumes of acetone; yield about 25 %, M.P. 54° and 63·0° respectively (54°, 63·1°).

Pentadecoic acid. 100 g. of Eastman Kodak acid (M.P. 48·5°) was fractionated five times by distillation *in vacuo* and after repeated crystallisation from light petroleum (B.P. under 40°) yielded 23 g. of pure acid; M.P. 52·5° (52·1°).

Stearic acid. Prepared by saponification of a specimen of ethyl stearate (iodine value 0·8) kindly supplied by Prof. Hilditch, which had been prepared by hydrogenation of ethyl oleate; M.P. 70·0° (70·1°).

Eicosanic acid. Prepared from Kahlbaum's erucic acid by heating with potassium hydroxide [Levene, West and Van der Scheer, 1915]. The crude acid was converted to the ethyl ester and fractionated at 206°/2·5 mm. After crystallisation from acetone the ester melted at 41–41·5° (41°) and the acid after repeated crystallisation from alcohol at 75–75·2° (75·2°).

Heneicosanic acid. Ethyl eicosanate was reduced to *n*-eicosanol by means of sodium in dry ethyl alcohol at 130°; yield 80 %, M.P. 65–65·5°. This was converted into the iodide (M.P. 41–42°) by heating with iodine and red phosphorus and hence to the nitrile by boiling overnight with potassium cyanide in absolute alcohol. Without isolation the nitrile was hydrolysed to heneicosanic acid by heating with excess of aqueous sodium hydroxide; yield 75 %; M.P. after repeated crystallisation from acetone and alcohol 74·2–74·6° (75·2).

Synthesis of ketones.

Symmetrical ketones. These have recently been prepared on a large scale in excellent yield by Grün, Ulbrich and Krczil [1926] who heated the requisite fatty acids in an iron vessel. In the present research the method of Gluud [1919] was first tried, as it seemed more applicable to small quantities of material. The barium soaps of the fatty acids were heated in a hard glass retort under reduced pressure, and the ketone, which slowly distilled over, was crystallised twice from alcohol. 12-Tricosanone (laurone), 15-nonacosanone and 16-hentriacontanone (palmitone) were prepared in this way in yields of 50–60 %. It was later found that improved yields could be obtained by a slight modification of the method of Grün *et al.* as follows.

Myristic acid (15 g.) was heated in a tall hard glass vessel to 285°. A loosely fitting sheet iron ring pierced with numerous holes had been previously placed in the vessel and was of such height that it was just covered by the molten acid. The melt was stirred by a glass paddle rotating within the ring, and after about 2 hours there was a sudden evolution of gas, with increase in viscosity of the liquid and considerable frothing. Heating was continued for a further 2 hours, when evolution of gas ceased. The melt was poured out of the tubes, cooled and then extracted with hot 95 % alcohol. The yellow solution was decolorised with charcoal, and on cooling the 14-heptacosanone (myristone) crystallised out. One recrystallisation from hot alcohol was sufficient to give a pure product; yield 9·7 g. or 75 %. 18-Pentatriacontanone (stearone) was also prepared by this method, the reaction temperature being 300–305°. The melting-points of these ketones and their oximes were in agreement with those of Grün *et al.* [1926] and of Channon and Chibnall [1929]. The oxime of 15-nonacosanone melted at 52–53°.

The following three unsymmetrical ketones were synthesised by the method used by Channon and Chibnall [1929] for the synthesis of 14-nonacosanone which is based on the synthesis of 6-ketostearic acid by Robinson and Robinson [1925]. The oxime of 14-nonacosanone has since been prepared; M.P. 45·5–46°.

12-Nonacosanone, $CH_3.(CH_2)_{10}.CO.(CH_2)_{16}.CH_3$. Ethyl palmitate, B.P. 152–155°/1 mm., was reduced with sodium in dry ethyl alcohol to *n*-hexadecanol, which was purified by distillation at 153–156°/1 mm., M.P. 50–51°. This was converted to the iodide by heating with iodine and red phosphorus. Sodium (5·5 g.) was dissolved in 90 cc. of absolute alcohol, ethyl acetoacetate (47 g.) added and finally *n*-hexadecyl iodide (82 g.). The mixture was boiled for 5½ hours, poured into 1500 cc. of water and the resulting ethyl 2-acetyl-*n*-octadecoate, $CH_3.(CH_2)_{15}.CHAc.CO_2.Et$, extracted with ether. It was fractionated twice and the fraction boiling at 189–195°/1·5 mm. collected; yield 56 g. (Found: C, 74·3; H, 11·8. $C_{22}H_{42}O_3$ requires C, 74·6; H, 11·9 %.) This ester (56 g.), dissolved in 300 cc. dry ether, was added to emulsified sodium (3·5 g.) suspended in 300 cc. dry ether and the mixture warmed until the sodium had dissolved. The mixture was then cooled on ice and dodecoyl chloride

(37·7 g.) in 80 cc. dry ether added. After standing for one hour the mixture was boiled for 10 minutes, cooled, the sodium chloride removed by washing with water and the ether removed by evaporation. The resulting oily 12-keto-13-acetyl-13-carbethoxy-*n*-nonacosane was dissolved without purification in 1400 cc. absolute alcohol. 5 % aqueous potassium hydroxide was then added until there was a slight turbidity and the mixture kept 4 days. Sufficient strong potassium hydroxide was then added to make the concentration 5 % and the solution boiled for 3 hours. The alcohol was evaporated and the solid residue extracted 6 times with hot acetone. The extracts on cooling deposited crystals, which were dissolved in light petroleum; the filtered solution was concentrated, and treated with two volumes of acetone. 12-Nonacosanone (20 g.) was precipitated. It was crystallised from 95 % alcohol (charcoal), M.P. 74·5°. (Found: C, 82·4; H, 13·3. $C_{29}H_{58}O$ requires C, 82·4; H, 13·8 %.) *Oxime*, M.P. 47–47·5°.

10-Nonacosanone, $CH_3.(CH_2)_8.CO.(CH_2)_{18}.CH_3$. By the method given above sodium (1 g.), alcohol (16 cc.), ethyl acetoacetate (7·5 g.) and *n*-octyl iodide (10 g.) gave 5·7 g. of ethyl 2-acetyl-*n*-decoate, a colourless oil, B.P. 140–141°/5 mm. Eicosanoyl chloride (6·4 g.) in dry ether (25 cc.) was added to ethyl sodio-2-acetyl-*n*-decoate (5·14 g.) in dry ether (25 cc.). The condensation product was saponified as before and yielded 2·4 g. of 10-nonacosanone. Recrystallised from light petroleum (B.P. 40–60°) it melted at 74·3–74·8°. (Found: C, 82·6; H, 13·8. $C_{29}H_{58}O$ requires C, 82·4; H, 13·8 %.) *Oxime*, M.P. 49·5–50°.

9-Nonacosanone, $CH_3.(CH_2)_7.CO.(CH_2)_{19}.CH_3$. Sodium (1 g.), alcohol (16 cc.), ethyl acetoacetate (7·5 g.) and *n*-heptyl iodide (10 g.) gave 6·3 g. of ethyl 2-acetyl-*n*-nonoate, a colourless oil, B.P. 122°/2 mm. Ethyl sodio-2-acetyl-*n*-nonoate (4·6 g.) condensed with heneicosanoyl chloride (6·3 g.) in dry ether yielded 2·2 g. of 9-nonacosanone. Recrystallised twice from light petroleum it melted at 73·5–74·2°. (Found: C, 82·2; H, 13·9. $C_{29}H_{58}O$ requires C, 82·4; H, 13·8 %.) *Oxime*, M.P. 48·5–49°.

All these long-chain ketones are fairly soluble in cold and very readily soluble in hot chloroform, benzene and xylene; slightly soluble in cold ethyl acetate, light petroleum (40–60°) and carbon disulphide; and almost insoluble in cold but fairly soluble in boiling acetone, ethyl and methyl alcohol. The oximes are readily soluble in cold light petroleum and in acetone-ether mixture.

Synthesis of secondary alcohols.

These were prepared in the usual way by reduction of the ketones. The ketone (1 g.) was dissolved in boiling amyl alcohol (40 cc.) and sodium (5 g.) slowly added. After 2 hours the mixture was cooled and washed with dilute hydrochloric acid and with water. The solvent was removed under reduced pressure and the residue recrystallised from alcohol (charcoal) and then from light petroleum (B.P. 40–60°). It was sometimes necessary to repeat the reduction. The melting-points (Table VI) of the symmetrical alcohols confirm

those of Grün *et al.* [1926]. The following have not previously been prepared. *15-Nonacosanol*, M.P. 83·6–83·8°, S.P.[1] 83°; *acetate* M.P. 50·5–51°. *14-Nonacosanol*, M.P. 79–79·3°, S.P. 78·4°; *acetate* M.P. 41–42°. *12-Nonacosanol*, M.P. 74–74·5°, S.P. 73°; *acetate* M.P. 44–45°. *10-Nonacosanol*, M.P. 74·8–75°, S.P. 74·2°; *acetate* M.P. 45–45·5°. *9-Nonacosanol*, M.P. 75·3–75·6°, S.P. 74·8°; *acetate* M.P. 44·5–45°.

The alcohols have similar solubilities to the ketones, but are markedly less soluble in cold solvents such as low-boiling petroleum.

Synthesis of higher paraffins.

The paraffins with an odd number of carbon atoms were synthesised by Clemmensen reduction of the corresponding ketones. The following synthesis of *n*-nonacosane, which has not hitherto been prepared, will illustrate the method employed.

n-*Nonacosane*. Zinc wool (30 g.) was amalgamated by allowing to stand in 5 % aqueous mercuric chloride for one hour. It was washed and placed in a flask fitted with a reflux condenser together with 15-nonacosanone (2 g.) and 200 cc. of a mixture of equal volumes of concentrated hydrochloric acid and alcohol saturated with dry hydrogen chloride. The mixture was then boiled until evolution of hydrogen slackened, when the flask was cooled and the aqueous alcohol poured off. Fresh acid mixture was added as many times as was necessary to dissolve all the zinc. A further 20 g. was then added and the operation repeated. The final product was washed with water and recrystallised from alcohol (charcoal). It melted at 62·7–63·0°, and analysis showed it to be a hydrocarbon. (Found: C, 85·2; H, 14·8. $C_{29}H_{60}$ requires C, 85·2; H, 14·8 %.) Paraffins prepared in this way were used in the earlier X-ray experiments, but the pictures obtained were inferior to those of natural paraffins. After various methods of purification had been tried it was found that treatment with sulphuric acid was the most effective. The paraffin was heated with about 6–8 volumes of concentrated sulphuric acid in a test-tube immersed in an oil-bath at 130° for 2 hours. Considerable blackening of the acid occurred. The tube was removed from the bath, the paraffin melt on the surface of the acid allowed to set, and the acid drawn off. Fresh acid was added and the tube replaced. This operation was repeated two or three times if necessary, depending on the amount of charring that occurred. The paraffin was washed repeatedly with hot water, and crystallised from 95 % alcohol (charcoal). The purified product was again treated with concentrated sulphuric acid, when it was usually found that a little darkening still occurred. The treatment was repeated until a sample of sulphuric acid which had remained in contact with the paraffin at 130° for 2 hours showed no darkening. In all cases this treatment raised the melting-point of the paraffin about 0·5°. There was usually a loss during purification of 10–20 %, due to the solubility of the paraffin in the acid and to the

[1] S.P. signifies setting-point.

decomposition of olefines formed during the reduction. (Before treatment with sulphuric acid the *n*-nonacosane had iodine value 1·3 and *n*-pentatriacontane 2·6.) We have proved by appropriate experiments that ketones and alcohols are also quantitatively removed by this treatment. The following are in general agreement with Grün *et al.* [1926] and Kraft [1882]: n-*heptacosane*, M.P. 59·0–59·2°, S.P. 58·5°; n-*hentriacontane*, M.P. 67·6–67·8°, S.P. 67·3°; n-*pentatriacontane*, M.P. 74·4–74·6°, S.P. 74·3°. An earlier sample of *n*-pentatriacontane prepared from another sample of stearic acid, which was thought at the time to be pure, gave the same M.P. (74·6°) but a lower S.P. (74·0°). The X-ray picture was also inferior. This sample was the one used by Garner *et al.* [1931].

The following paraffins were synthesised by the method of Gascard [1921].

n-*Octacosane.* This paraffin has not previously been synthesised. A sample of *n*-tetradecanol was kindly supplied by Prof. Garner. It had been twice distilled, then recrystallised four times from alcohol and finally distilled, M.P. 38–38·5°, S.P. 37·7°. It was converted into the iodide (2·17 g.) which was boiled in sulphur-free xylene (70 cc.) with sodium (0·2 g.) for 3 hours. The cooled solution was washed with water, evaporated to 10 cc. and treated with two volumes of alcohol to precipitate the paraffin (0·54 g. or 36 %). The first treatment with sulphuric acid for 4 hours at 130° caused a little blackening of the acid, the second none at all, M.P. 61·4–61·5°, S.P. 61·1°. This paraffin was also prepared by the method of Backmann and Clark [1927]. Tetradecyl iodide (2·46 g.) was heated at 150° for 2 hours and sodium (1·3 g.) added slowly in small pieces with vigorous stirring. The yield after treatment with sulphuric acid was 70 % of theory. The X-ray picture showed that this preparation was inferior to the former, although the M.P. and S.P. were the same.

n-*Dotriacontane.* *n*-Hexadecyl iodide (1·13 g.) prepared from synthetic *n*-hexadecanol (p. 2074) was boiled in sulphur-free xylene (50 cc.) with sodium (0·2 g.) for 4 hours and the paraffin treated as before; yield 0·16 g., M.P. 69·5–69·7°, S.P. 69·3°. The paraffin prepared from purified natural cetyl alcohol (M.P. 49°) had M.P. 70·3–70·7°, confirming that of Gascard [1921], 70·5°. Kraft [1882] pointed out that natural cetyl alcohol contained some 4–5 % of octadecyl alcohol; the M.P. of the derived paraffin confirms this.

n-*Hexatriacontane.* This was prepared from *n*-octadecyl iodide (0·5 g.) kindly supplied by Dr Malkin, and sodium (0·1 g.) in boiling xylene as before, M.P. 75·7–75·9°, S.P. 75·4°. (Gascard [1921], M.P. 76°.)

The following paraffins were kindly supplied by Prof. Garner [*cf.* Garner, van Bibber and King, 1931]. They had been prepared by electrolysis of the respective fatty acids [Peterson, 1906] and were purified by treatment with sulphuric acid.

n-*Hexacosane*, M.P. 56·4–56·6°, S.P. 56·1° (Gascard [1921], M.P. 56·5°; Garner *et al.* [1931], S.P. 56·1°).

n-*Triacontane*, M.P. 65·6–65·8°, S.P. 65·5° (Gascard [1921], M.P. 65·6–66°; Garner *et al.* [1931], S.P. 65·6°). X-ray analysis showed that this paraffin was not pure, although the M.P. seems correct.

n-*Tetratriacontane*, M.P. 72·5–72·8, S.P. 72·4 (Gascard [1921], M.P. 73·2°; Garner *et al.* [1931], S.P. 72·4°).

These long-chain paraffins are freely soluble at room temperature in low-boiling light petroleum and carbon disulphide [*cf.* Brooks, 1922, p. 581 for solubilities of paraffin wax] but the solubility decreases rapidly with lowering of temperature, and with increase of molecular weight.

For the carbon and hydrogen analyses of the substances mentioned in this paper the usual micro-apparatus of Pregl [1924] with the phosphorus pentoxide absorption tube of Drew and Porter [1928] was first used. The carbon, especially in the case of paraffins, was frequently low, possibly due to the volatility of the substances at high temperature. To obtain accurate and reproducible results it was necessary to modify the apparatus and usual procedure in the following way. The length of the copper oxide column in the tube was increased from 140 mm. as recommended by Pregl to 160 mm., the gauze round that part of the tube containing this oxide being heated to a bright red heat. The actual burning of the compound was carried out rather more slowly than is necessary for the average substance, 70 cc. of oxygen being passed in for this purpose at the rate of not more than 3 cc. per minute, this being followed for "sweeping out" purposes by 100 cc. of air at 4–5 cc. per minute.

Measurement of the crystal spacings.

There are now available large numbers of measurements of the 001 spacings of various long-chain compounds. For any homologous series linear relationships exist between these spacings and the number of carbon atoms in the chain. It is obvious that the spacings of a series once measured will serve as a means of identification. Original work on synthetic paraffins has been carried out by Müller and Savile [1925, 1928, 1930] and Piper *et al.* [1925].

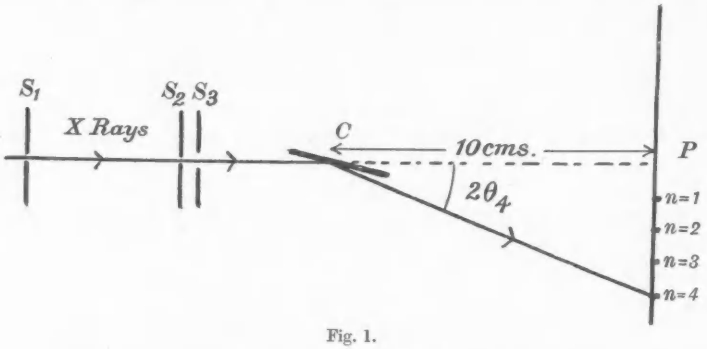


Fig. 1.

Although the technique has often been described it will be convenient to outline the procedure. The substances all crystallise in flakes, and the required planes lie parallel to the flake surfaces. A very thin layer is pressed on to a plate-

glass mount to give a surface of about 1 cm. by 5 mm. wide. The plate is held vertically in a geometric clamp on the spectrograph in such a way that the surface is in the axis of rotation of the table. A narrow beam of X-rays from a Shearer tube of copper anticathode, filtered by a nickel foil, passes through the slits S_1, S_2, S_3, Fig. 1, and falls on the crystals at grazing incidence. The crystal is rocked to and fro through 14° by a cam mechanism, and the radiation it reflects is received on a photographic plate placed 10 cm. away at P. This plate is adjustable for height and is provided with a lead screen allowing successive exposures of strips 5 mm. deep. As many as 16 exposures can be made on the same plate. An exposure takes 30 minutes after which the crystal is turned through 180° and a second exposure is made on the other side. Reflection only occurs at glancing angles θ_1, θ_2, θ_3, *etc.*, determined by the Bragg equation

$$n\lambda = 2d \sin \theta_n,$$

where λ is the wave-length, $n = 1, 2, 3$, *etc.*, the order of the reflection, and d the crystal spacing. With good material and chains of 20 or more carbon atoms 9 or 10 orders can be obtained between $\theta = 0$ and 14°. The trace on the plate consists of a series of lines approximately equally spaced and decreasing in intensity with increasing order, and, since $\sin \theta$ is inversely proportional to d, the lines on the plate are close together for large spacings (long chains) and more widely separated for short chains. The values of θ for as many orders as possible are found, and d calculated. It has to be remembered that the actual position of the reflecting surface is indeterminate, but in front of the axis of rotation of the table. The correction to be applied decreases with increasing order and must be found by successive approximations from the value of d obtained from a high order. As a result the larger the number of orders measured the greater the accuracy attainable.

The spacing of a given specimen of paraffin can, with the apparatus in use, be measured to an accuracy of 0·1 %. However, we have found that different synthetic preparations of the same paraffin, apparently equally pure, and made by the same process, sometimes do not give spacings agreeing to less than 0·2 %. A repetition of the spacings to an accuracy of 0·1 % can probably only be obtained by working with single crystals, and we have not found it profitable to attempt more than 0·25 % except for the synthetic standards.

A spacing-carbon atoms graph for the paraffins is shown in Fig. 2. The points for 20, 21 and 24 carbon atoms are taken from Müller and Savile [1930], the higher members have been freshly determined from the material whose preparation has been described and are shown in Table I. Fig. 2 shows that the paraffins are polymorphous; *n*-hexacosane, for instance, may crystallise in two forms having spacings 34·95 and 31 Å. The particular crystal form adopted depends upon temperature, the length of the chain, and the degree of purity of the specimen.

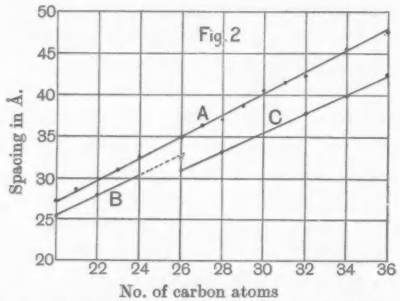
Odd number paraffins of 11 or more carbon atoms normally crystallise with the chain axis vertical to the 001 planes, and the planar spacing is a direct

measure of the length of the molecule. Even number paraffins of 18 or more atoms crystallise in this form at temperatures near the melting-point. These spacings fall on line *A* of Fig. 2. Even number paraffins up to 24 atoms, at normal temperatures, crystallise with the chain axis inclined at a constant angle to the planes. The 001 spacings of this *B* modification are shorter than those of the *A* form and lie on another straight line. *B* crystals are converted to *A* at temperatures very close to the melting-point and resume the *B* form on cooling. Odd number chains of 9 or less also adopt the *B* form under similar conditions [Müller and Savile, 1930].

Table I. *Melting-points and crystal spacings of the paraffins.*

Paraffin	First transition-point on heating	Second transition-point on heating	M.P.	S.P.	First transition-point on cooling	Second transition-point on cooling	Series No.	Crystal spacing in Å. *A*	*B*	*C*	
$C_{26}H_{54}$	51·5–52°	56·0°	56·4–56·6°	56·1°	55·5°	48·3°	C 67	34·95	32·6	31	*B* has only appeared in natural products
$C_{27}H_{56}$	52·8–53°	58·8–58·9°	59·0–59·1°	58·5°	58°	51°	C 22, 23	36·38	—	—	
$C_{28}H_{58}$	57–57·4°	61·2°	61·4–61·5°	61·1°	60·4°	54°	C 114	37	—	33·25	
$C_{29}H_{60}$	57·3–57·5°	63·2°	63·4–63·6°	63·2°	62·2°	55·8°	C 26	38·68	—	—	
$C_{30}H_{62}$	59–59·5°	65–65·3°	65·6–65·8°	65·4°	64·3°	58°	C 69	40·5	—	Not observed	Impure
$C_{31}H_{64}$	62–62·5°	67·2–67·4°	67·6–67·8°	67·3°	66·2°	61·8°	C 125	41·55	—	—	
$C_{32}H_{66}$	65·2–65·4°	69·2–69·4°	69·5–69·7°	69·2°	68·6°	63·9°	C 122	42·33	—	37·8	
$C_{34}H_{70}$	69·2–69·4°	72·2°	72·6–72·8°	72·4°	71·4°	68·5°	C 70, 71	45·5	—	39·86	
$C_{35}H_{72}$	71·8–72°	74·0°	74·4–74·6°	74·1°	73·0°	70·5°	C 28	46·64	—	—	
$C_{36}H_{74}$	73·9–74·1°	75·1–75·3°	75·7–75·9°	75·6°	74·6°	72·5°	B 121	47·5	—	42·33	

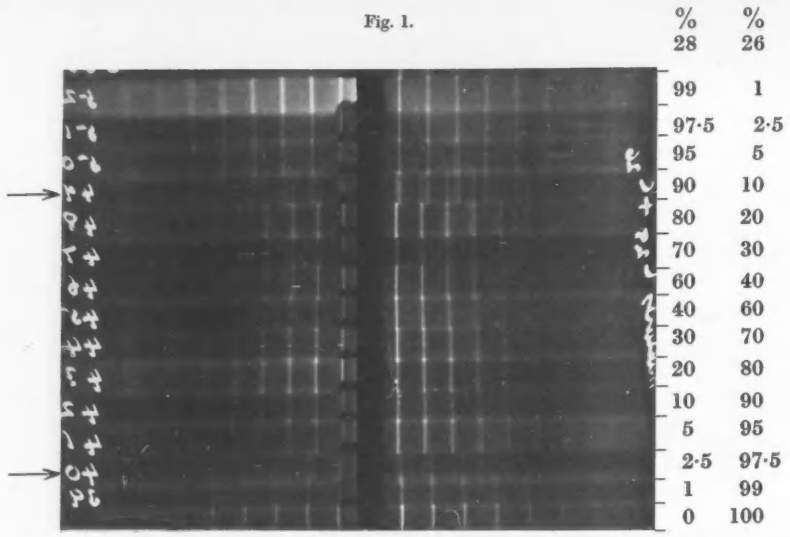
The *A* spacings of the even number paraffins are not so accurate as the remaining measurements. They only occur at temperatures near the M.P. for good crystals, and it is difficult to measure more than three orders.



× = This spacing has not been observed in synthetic hexacosane.

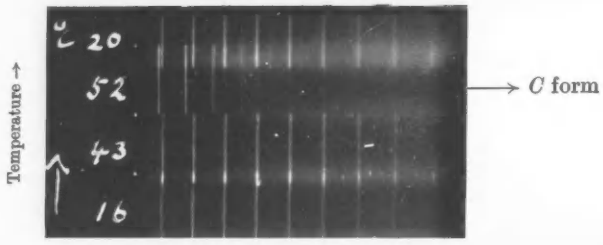
The paraffins that have been most important in this work have 26 or more carbon atoms. If pure these crystallise stably in yet a third modification, *C* of Fig. 2. This form is characterised by a larger tilt of the molecular axis and, of course, by still shorter spacings [Piper and Malkin, 1930]. *n*-Hexacosane can apparently appear in all three forms. We are mainly concerned with spacings lying on the *A* and *C* lines, these are given in Table I. *C* form is converted to *A* at a temperature about 5° below the M.P. Of our specimens hexacosane

Fig. 1.



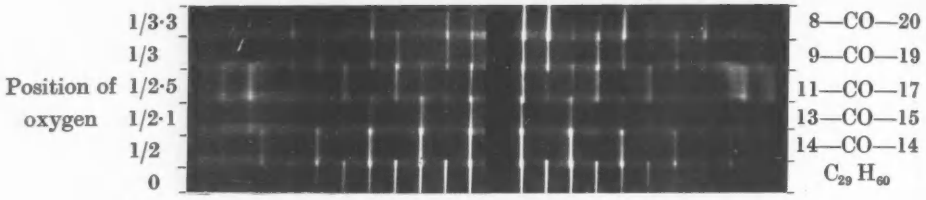
Synthetic mixtures. Changes from *A* to *C* form marked by arrows.

Fig. 2.



Temperature change in *n*-hexacosane.

Fig. 3.

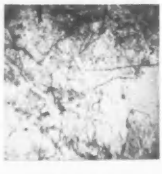


Shearer intensity variation in some nonacosanones.

Fig. 4.

Tetratriacontane.
Polarized light.

Fig. 5.



Triacontane.

Fig. 6.

Tetratriacontane.
Unpolarised light.

reverted to *C* on cooling, the others did not, and the reverse change, if it occurs, must be extremely slow at room temperatures. A photograph of hexacosane in both forms is given in Plate VIII, Fig. 2.

The melting- and transition-points of the paraffins.

If the melting-point of a paraffin is carefully determined, as described below, it is possible to obtain not only the true melting- and setting-points but also to determine two transition-temperatures at which a change in crystalline form occurs. The lower of these transition-temperatures is markedly affected by impurities and, in certain cases, is a much more useful guide to the purity of the paraffin than the melting- or setting-point itself. It is necessary therefore to describe in some detail our method of taking these temperatures.

The melting-point apparatus was of the usual glass bulb type and held 80 cc. of sulphuric acid. It was fitted with a standard short stem thermometer which had a spherical bulb of diameter 4 mm. and was graduated in 0·2°. The acid in the bulb was not stirred, and, by using a small flame, it was possible with care to repeat a melting-point with an error of $\pm$ 0·1°. The material was first melted into open capillary tubes of outside diameter about 1 mm. The depth of the melt, which, after sealing, was jerked sharply to the bottom of the tube, was about 2 mm. All melting-points are corrected.

The following observations for *n*-nonacosane will illustrate the method of taking melting-points. The melting-point tube lying alongside the bulb of the thermometer is viewed through a lens giving a magnification of $\times$ 3. At room temperature the material in the tube appears as a white opaque crystalline mass. The temperature is raised rapidly to about 55° and then more slowly (1° per minute). At 57·3–57·5° a transition occurs. The crystalline structure disappears, the material becomes colourless, and an air gap down the centre, in the form of an attenuated inverted cone, is revealed. The temperature is again raised fairly rapidly to 63° and then, still more slowly than before, through the final melting-point. At 63·2° the material once again becomes opaque and the cone structure is no longer seen. Fusion takes place at 63·4–63·6° and the temperature is held within these limits until all the material is melted. The temperature is then allowed slowly to fall; at 63·2° long needles appear throughout the whole molten mass. The heating flame is then withdrawn so that the bath cools a little more rapidly. Between 62·5° and 62·2° the crystalline structure disappears. Contraction during the change of state leads to the formation of the inverted cone-shaped air gap, the rest of the material becoming clear and colourless. At 56° transition takes place quite suddenly to the original white crystalline and opaque form, which is the stable one at room temperatures.

In the case of the longer-chain paraffins, $C_{34}H_{70}$, $C_{35}H_{72}$ and $C_{36}H_{74}$, the transitions are much less marked, and can only be observed if the previous cooling of the material in the tube has been very slow. It is the usual practice in our laboratory to place the tube, when first made, in a bath which is at a

temperature already above the melting-point, and to take the setting- and transition-points on cooling. The melting- and transition-points on heating are taken later. The time required to make the six observations is about 25–30 minutes. To eliminate the personal factor all the melting- and transition-points recorded in this and the two following papers were taken by one observer (A. C. C.).

The melting-, setting- and transition-points of the paraffins between $C_{26}H_{54}$ and $C_{36}H_{74}$ are given in Table I. As we believe that all these compounds (except $C_{30}H_{62}$) are pure the following deductions can be made.

Melting-points. The series $C_{26}H_{54}$ to $C_{32}H_{66}$ lie on a smooth curve (Fig. 3). There is evidence of alternation between $C_{34}H_{70}$, $C_{35}H_{72}$ and $C_{36}H_{74}$. We are therefore not entirely in agreement with the conclusions of Hildebrand and Wachter [1929] who adopted the somewhat dubious course of plotting all the values to be found in the literature, which assumes that all the products have been made from fatty acids of equal purity. The M.P. determined by our method may be some 0·5–0·7° lower than that given by the more usual method in which the temperature of a stirred bath is raised from 3° to 6° per minute. Even so we are unable to explain the high values recorded by Levene, West and Van der Scheer [1915].

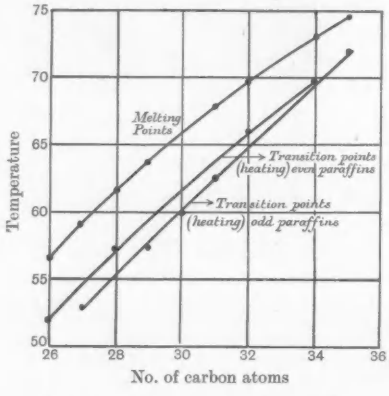


Fig. 3. Melting- and transition-points.

Setting-points. Supercooling was never observed in the case of pure paraffins or mixtures of pure paraffins, and it is unnecessary to leave a nucleus.

First transition-point. Alternations between odd and even numbered chains are observed in the change from the stable low temperature crystalline form to the colourless glass. The temperatures recorded are few in number, but give satisfactorily concordant results. The values are plotted in Fig. 3. Both odd and even series show a convergence of melting- and transition-points as the

chain length increases. Particularly interesting is the position of the transition-temperature of triacontane which falls on the odd number paraffin line. This particular specimen of triacontane will only crystallise with a vertical chain (the *A* form), and, in view of the fact that this is the crystal modification adopted by the odd number paraffins, its position among them appears normal. The even number paraffins crystallising normally with a tilted molecule show, as would be expected (since for them the vertical chain is a less stable form), higher transition-temperatures (Fig. 3). All transition-temperatures on cooling lie on one smooth curve.

Second transition-point. This is often difficult to observe and as it is not apparently affected by impurities it will not be quoted in the tables dealing with mixtures and in further publications. In the future, "transition-point" is to be taken as referring to the first transition mentioned above.

Crystal forms of paraffins.

Microscopic investigation confirms the melting-point data, namely that all the longer paraffins have a transition-point involving a structural change. Plate VIII, Figs. 6 and 4, shows the appearance of some very pure *n*-tetratriacontane under the microscope and between crossed Nicols. The crystals, which are in the *C* modification, appear as rhombs of acute angle 73° and show very clear straight cleavages. There is also a marked double refraction effect noticeable. Plate VIII, Fig. 5 is a specimen of *n*-triacontane in unpolarised light; it is not quite pure and does not display such marked cleavages, also the crystals are very much smaller. They show no marked effect between crossed Nicols. These crystals are in the *A* form. Plate VIII, Figs. 5 and 6 should be compared as showing the difference in appearance in unpolarised light of a fair and a very good preparation of an even number paraffin. Odd number paraffins never take the *C* form, but Müller and Savile [1928] has found single crystals in the *A* form with an acute angle of 66°. The odd number paraffins examined had no definite optic axes, but the crystals were extremely minute and in a confused mass, and there was indication of a slight restoration of the light through crossed Nicols. If the crystals are placed in a heating stage they will all be found to undergo a structural change about 5° below the M.P. both on heating and cooling. A crystal in the *C* form breaks up at this temperature and does not appear to recrystallise with its previous optical properties.

The apparent lack of concordance between these observations and the X-ray data, which indicate transitions only for even number paraffins, is due to the fact that the latter, in our case, deal only with one crystal dimension. The reversible change common to all appears to correspond with one observed by Müller and Savile [1930] near the M.P., and presumably involves a change of cross-section of the cell without affecting the molecular tilt. Such a change should not affect the spacings we photograph. But with the longer even members there is, in addition, a change in tilt at or near this temperature which does not always reverse on cooling, and this change involves an increase in the 001

spacing. A very similar temperature change is found with even number fatty acids, Piper *et al.* [1926].

The heats of crystallisation of the paraffins of 22, 26, 30, 34 and 35 carbon atoms have been measured by Garner, van Bibber and King [1931]. Temperature transitions were found for all these at temperatures about 5° below the M.P. Now it is noticeable that while the heats of transition of 22, 26 and 34 increase uniformly with chain length, the values for 30 and 35 are lower. Prof. Garner kindly allowed us to photograph his material and we found that 22, 26 and 34 were in *B* or *C* form whilst 30 and, of course, 35, were in the *A* modification. Thus both paraffins with a low heat of transition had vertical chains and the other three tilted chains. The spacing and M.P. of triacontane were both high, which suggests contamination by not less than 2·5 % of a longer-chain paraffin. Impurity may not have a marked direct effect on the heat of transition, but it tends to force an even number paraffin to crystallise with a vertical chain, and it is plausible to suggest that part at least of the defect in heat of transition for triacontane represents the difference in energy between the *C* and *A* forms of this paraffin. *C* will, of course, have slightly less energy. Throughout their curves there is no indication of a double break, so the change in tilt occurs simultaneously with the other structural change.

Spacings of mixtures of paraffins.

Assuming that the anomalous results obtained with *n*-triacontane (low heat of transition, anomalous transition temperature, no *C* spacing) were due to lack of purity, we attempted a systematic investigation of the crystallisation of mixed paraffins. These were prepared in the following way. Benzene solutions of known strength were made for each paraffin. To prepare the necessary mixture the requisite aliquot of each benzene solution was pipetted into a small beaker, the benzene removed almost completely on a water-bath, and two volumes of hot alcohol were added. The paraffin mixture crystallises out almost quantitatively on cooling. Since the paraffins have a small residual solubility in the mixed solvent it follows that when one is added only in small amount, *e.g.* 1 %, the mixed crystals may actually contain a little less than this proportion. It is known from recent work on the separation of mixed paraffins by fractional crystallisation that temperature of crystallisation can slightly affect the composition. The spacings for binary mixtures whose chain-lengths differ by one or two carbon atoms are shown in Tables II–V and Figs. 4–7.

Only one 001 spacing occurs for all mixtures unless the components are separated by at least 4 carbon atoms. We commenced with two odd number chains (29 and 31) as the results are not confused with alternative crystal forms. A 1 % addition of 31 in 29 raises the spacing by about 1 %. The spacing continues to rise fairly uniformly with the amount of 31 until at equimolar proportions it is 41·2, approximately the spacing of the *n*-hentriacontane used. The mixed spacing continues to rise to a maximum when 97·5 % of the higher paraffin is present and then falls to that of pure *n*-hentriacontane. This behaviour

appears general for A form spacings; except in one case paraffin impurity up to 1 % increases the spacing whether the contaminating chain is shorter or longer. For 10 % of a shorter chain the spacing approximates to that of the pure substance, but the number of orders decreases. With a longer contaminating chain

Table II. *Melting-points and crystal spacings of mixed paraffins.*

($C_{28}H_{58}$ and $C_{29}H_{60}$.)

Percentage composition						X-ray analysis			
		Transition-point on heating	M.P.	S.P.	Transition-point on cooling	Series number	Spacing in Å.		Remarks
$C_{28}H_{58}$	$C_{29}H_{60}$						*A*	*C*	
100	0	57·2–57·4°	61·3–61·5°	61·1°	53·7°	C 114	37	33·25	The *C* form vanishes at contamination 2·5 %
99	1	56·2°	61·2–61·5°	61·1°	53·2°	B 63	—	33·25	
97·5	2·5	56·0°	61·2–61·5°	61·1°	53·4°	B 64	—	33·3	
95	5	56·0°	61·3–61·5°	61·1°	53·6°	B 65	37·55	—	
90	10	55·2°	61·5–61·7°	61·3°	54·2°	B 93	37·8	—	
80	20	55·0°	61·9–62°	61·5°	53·6°	B 92	38	—	
70	30	55·0°	62·1–62·2°	61·7°	53·5°	B 91	37·85	—	
60	40	55·2°	62·3–62·4°	62·0°	53·8°	B 90	38·1	—	
50	50	55·6°	62·3–62·6°	62·3°	54·0°	B 89	38·5	—	
40	60	55·6°	62·6–62·8°	62·4°	54·2°	B 88	38·45	—	
30	70	56·2°	62·8–62·9°	62·5°	54·4°	B 87	38·45	—	
20	80	56·2°	63–63·2°	62·8°	54·7°	B 86	38·6	—	
10	90	56·8°	63·2–63·4°	63·0°	55·2°	B 62	38·7	—	
5	95	57·2°	63·2–63·5°	63·1°	55·4°	B 61	38·7	—	
2·5	97·5	57·5°	63·3–63·5°	63·2°	55·6°	B 60	38·9	—	
1	99	57·5°	63·4–63·6°	63·2°	55·8°	B 59	38·8	—	
0	100	57·5°	63·4–63·6°	63·2°	56·0°	C 26	38·68	—	

Table III. *Melting-points and crystal spacings of mixed paraffins.*

($C_{29}H_{60}$ and $C_{31}H_{64}$.)

Percentage composition		Transition-point on heating	M.P.	S.P.	Transition-point on cooling	X-ray analysis		Remarks
						Series number	Spacing in Å.	
$C_{29}H_{60}$	$C_{31}H_{64}$							
100	0	57·3–57·5°	63·4–63·6°	63·2°	55·8°	C 26	38·68	
99	1	57·2–57·4°	63·4–63·6°	63·2°	55·4°	C 106	39·12	
97·5	2·5	57–57·5°	63·5–63·7°	63·2°	55·4°	C 96	39·25	
95	5	57–57·2°	63·6–63·7°	63·1°	55·3°	C 97	39·15	
90	10	56·5–57°	63·8–64°	63·3°	55·2–55°	C 98	39·45	
85	15	56·0–56·5°	64–64·2°	63·5°	55–54°*	C 101	39·7	
80	20	56·0–56·5°	64·2–64·4°	63·7°	55–54°*	C 102	39·75	
70	30	56·5–57°	64·6–64·7°	64·1°	55–54°*	C 103	40·55	
60	40	56·5–57°	64·8–65°	64·4°	55–54°*	C 104	40·9	
50	50	57–57·5°	65·3–65·5°	65°	55·5–55°*	C 111	41·2	
40	60	57·5–58°	65·8–66°	65·5°	56–55°*	C 110	41·2	
30	70	59–59·5°	66·2–66·4°	65·8°	57·5–57°*	C 109	41·45	
20	80	60–60·5°	66·6–66·8°	66°	58·5°*	C 105	41·5	
10	90	61–61·5°	67·1–67·3°	66·8°	59·5°	C 108	41·7	
5	95	61·7–61·9°	67·3–67·5°	67·1°	60°	C 100	41·85	
2·5	97·5	61·7–61·9°	67·4–67·6°	67·3°	61·6°	C 99	41·8	
1	99	62·1–62·3°	67·6–67·7°	67·3°	61·7°	C 107	42·0	This increase in spacing is greater than the possible experimental error
0	100	62·3–62·5°	67·6–67·8°	67·3°	61·8°	C 32	41·55	

*=indefinite.

the *A* spacing increases steadily up to 80 % impurity. The *A* spacing then is very sensitive to the addition of small amounts of other paraffins. With *n*-hexacosane and *n*-octacosane mixtures, since both have even number chains, we have to deal with two crystal forms. Both crystallise normally in the *C* form but if more than 2·5 % of the longer chain is added to the shorter, crystallisation occurs only in the *A* form. Up to 10 % of a short chain can be added to a long

one before the *C* modification fails to appear. This is clearly shown in Plate VIII, Fig. 1. The tables show that this is a true generalisation whether the contaminating paraffin has an odd or an even number chain. There is no measur-

Table IV. *Melting-points and crystal spacings of mixed paraffins.*

($C_{26}H_{54}$ and $C_{28}H_{58}$.)

Percentage composition						X-ray analysis			
							Spacing in Å.		
$C_{26}H_{54}$	$C_{28}H_{58}$	Transition-point on heating	M.P.	S.P.	Transition-point on cooling	Series number	*A*	*C*	Remarks
100	0	51·5–52°	56·4–56·6°	56·1°	48·3°	C 67	34·95	31	*C* spacing disappears at more than 2·5 % impurity
99	1	51°	56·5–56·6°	56·0°	48·0°	B 39	—	31	
97·5	2·5	51°	56·5–56·7°	56·0°	47·4°	B 40	35·1	31·6	
95	5	50°	56·5–56·8°	56·0°	47·0°	B 41	35·5	—	
90	10	48·5–49·5°	56·7–57·0°	56·3°	46·5°*	B 42	35·25	—	
80	20	48·5–49·5°	57·2–57·5°	57·0°	46·5°*	B 43	36	—	
70	30	48·5–49·5°	57·8–58°	57·5°	46·5°*	B 44	36·2	—	
60	40	48·5–49·5°	58·4–58·6°	58·2°	47·0°*	B 45	36·55	—	
50	50	49–50°	58·9–59·1°	58·5°	47·4°*	B 97	36·7	—	
40	60	49–50·5°	56·6–56·7°	59·2°	48·8°*	B 46	36·8	—	
30	70	51–52°	60·1–60·2°	60·0°	50·6°*	B 47	37·7	—	
20	80	52–53°	60·4–60·6°	60·1°	52·5°	B 48	37·4	—	
10	90	53–53·4°	61·0–61·1°	60·4°	52·7°	B 49	37·55	33·45	
5	95	54·5°	61·1–61·3°	60·9°	53·2°	B 50	—	33·35	*C* spacing disappears at more than 10 % impurity
2·5	97·5	55–55·5°	61·2–61·4°	60·9°	53·3°	B 51	—	33·3	
1	99	56·5–57°	61·2–61·5°	60·9°	53·4°	B 52	—	33·3	
0	100	57·4°	61·3–61·5°	61·1°	54·0°	C 114	37	33·26	

* = indefinite.

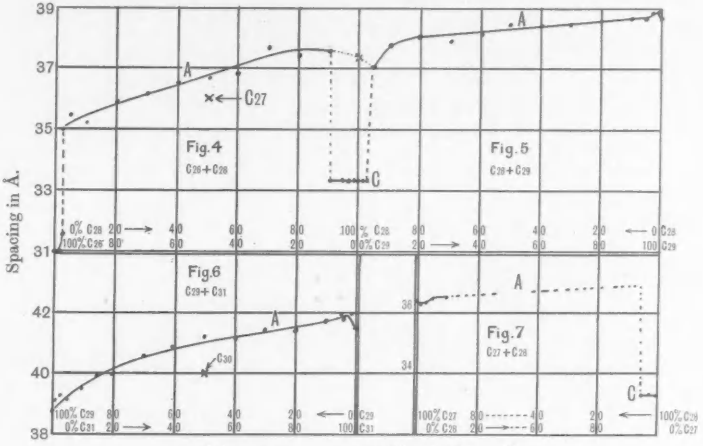
Table V. *Melting-points and crystal spacings of mixed paraffins.*

Composition (equimolar unless stated)	Transition-point on heating	M.P.	S.P.	Transition-point on cooling	Series number	Crystal spacing in Å. *A*	Crystal spacing in Å. *C*	Remarks
97·5 % C_{26}+2·5 % C_{27}	49·4–49·6°	56·4–56·6°	56·1°	48°	B 100	—	30·9	
97·5 % C_{27}+2·5 % C_{26}	52·5–53°	59·0–59·1°	58·6°	—	B 99	36·15	—	
99 % C_{27}+1 % C_{28}	52·5–53°	59·0–59·1°	58·7°	50·6°	B 55	36·3	—	
97·5 % C_{27}+2·5 % C_{28}	52·5–53°	59·0–59·2°	58·8°	—	B 56	36·3	—	
95 % C_{27}+5 % C_{28}	52·5–53°	59·1–59·3°	58·9°	50°	B 57	36·45	—	
90 % C_{27}+10 % C_{28}	52·5–53°	59·2–59·5°	58·9°	50°	B 58	36·55	—	
99 % C_{28}+1 % C_{27}	56·8°	61·2–61·4°	61·1°	53·4°	B 66	—	33·2	
97·5 % C_{28}+2·5 % C_{27}	56·4°	61·2–61·4°	61·0°	53·3°	B 67	—	33·3	
95 % C_{28}+5 % C_{27}	55·6°	61·2–61·4°	61·0°	53·2°	B 68	—	33·2	
90 % C_{28}+5 % C_{27}+5 % C_{29}	55·2°	61·3–61·5°	61·2°	—	B 69	37·7	—	
90 % C_{28}+5 % C_{26}+5 % C_{30}	54–54·4°	61·3–61·5°	61·2°	—	B 70	38·0	—	
97·5 % C_{32}+2·5 % C_{31}	64°	69·4–69·6°	68·8°	63·4°	B 98	—	42·5	
C_{27}+C_{28}+C_{29}	53·4°	61·0–61·4°	60·8°	—	B 54	38·25	—	
C_{26}+C_{28}+C_{30}	50–51°	61·6–61·8°	61·3°	—	B 71	—	—	
C_{26}+C_{27}+C_{28}+C_{29}+C_{30}	44°	61–61·5°	60·7°	—	B 79	—	—	
C_{27}+C_{29}+C_{31}	52·3°	63·5–63·6°	62·8–63°	—	C 86	40·2 37·75	—	
C_{29}+C_{30}+C_{31}	58–59°	65·6–65·7°	65·4°	—	C 84	40·8	—	
C_{31}+C_{35}	55°	70·7–71·2°	70·3–70·5°	—	C 82/3	46·25 43·0	—	Approximate to the spacings of the constituents
C_{24}+C_{25}	69°	73·6–73·9°	73·3°	—	C 89	46·05	—	

able change in *C* spacing until the *A* form appears, spacing variations then follow the course described for two odd number paraffins.

A study of Plate VIII, Fig. 1, reveals marked differences in the intensity decrease with increasing order as the mixtures approach equimolar proportions. Little radiation is reflected beyond the fourth order, and the four orders that

do appear stand out much more sharply than in the plates of the more nearly pure materials. To make such a comparison fairly it is of course necessary to take the whole range of photographs under identical conditions. An experienced eye can tell from a glance at a photograph whether or not a given specimen is approaching purity, though in the case of odd number paraffins which have no *C* form, it is easy to overestimate the degree of purity attained.



× = X-ray measurements with mixed paraffins

Figs. 4, 5, 6 and 7.

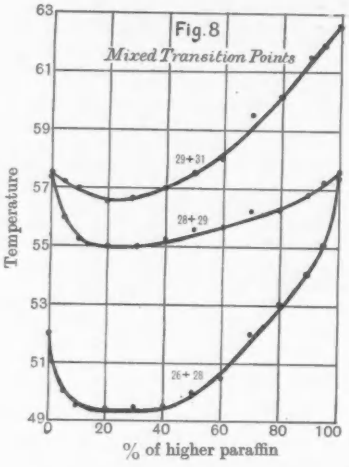
A few complicated mixtures have been examined, but X-ray data lead to no important generalisations. It will however be observed that in equimolar binary mixtures of chains that differ by 4 or more carbon atoms, distorted crystals of both constituents separate, though in the case of octacosane and dotriacontane a melted layer gave only one spacing. In this connection it may be noted that the *A* spacing of octacosane is 37 and the *C* value of dotriacontane 37·8. The mixed crystal from the melt may consist of tilted 32 and upright 28 molecules though the value found, 40 Å., is high.

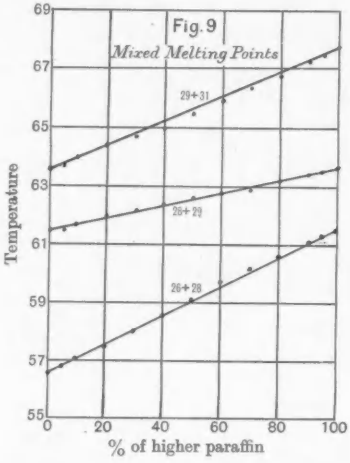
The melting- and transition-points of mixtures of paraffins.

The melting-, setting- and transition-temperatures of a comprehensive series of mixed paraffins are given in Tables II–V. It is proposed to summarise here only those observations which will aid an observer in judging the purity of a given sample of a paraffin.

Melting-point. The melting-points of mixtures of two paraffins given in Tables II–IV lie in each case on straight lines, see Fig. 9. There is no depression similar to that observed with the fatty acids, alcohols or ketones, so that (for instance) equimolar $C_{26}H_{54}+C_{28}H_{58}$ melts at the same temperature as $C_{27}H_{56}$ (59·1°) and equimolar $C_{29}H_{60}+C_{31}H_{64}$ (65·6°) the same as $C_{30}H_{62}$ (65·7°). In the

case of equimolar mixtures of three paraffins, the melting-points are still quite sharp and correspond with the mean molecular weight. In the case of equimolar mixtures of four or more paraffins the melting-points are indistinct, but are in the region corresponding with the mean molecular weight. To give a concrete example the following paraffins and mixtures all melt at approximately the same temperature (61·4–61·6°): $C_{28}H_{58}$, equimolar $C_{27}H_{56}+C_{29}H_{60}$, $C_{28}H_{58}+5\ \%\ C_{27}H_{56}+5\ \%\ C_{29}H_{60}$, $C_{28}H_{58}+5\ \%\ C_{26}H_{54}+5\ \%\ C_{30}H_{62}$, equimolar $C_{26}H_{54}+C_{28}H_{58}+C_{30}H_{62}$, equimolar $C_{26}H_{54}+C_{27}H_{56}+C_{28}H_{58}+C_{29}H_{60}+C_{30}H_{62}$. It is obvious from these results that the purity of a paraffin cannot be judged by a comparison of melting-point, or by mixed melting-point with a sample of a paraffin of known purity.





Setting-points. In the case of equimolar mixtures of two or three paraffins whose carbon content does not differ by more than two or three carbon atoms, the setting-points are quite sharp and are not appreciably depressed. In the case, however, of a mixture of, say, two paraffins differing by three or more carbon atoms it will be found that the melting-point extends over a somewhat broad range, 0·5–0·7°, and that the upper limit of the setting-point may be higher than the lower limit of the melting-point. This is due to the separation of quite large crystal masses containing an excess of the higher melting constituent. The fact that the setting-point is very close to the melting-point, therefore, is not necessarily a guarantee that the paraffin is pure. Equimolar mixtures containing more than three paraffins do not set crystalline; instead a white dense mist slowly separates from the bottom of the tube upwards.

Transition-points. As we believe that the transition-point alone can serve as a satisfactory guide throughout the separation of a mixture of naturally

occurring paraffins, these call for more extended comment. In the case of mixtures of neighbouring paraffins, for example $C_{28}H_{58}$ and $C_{29}H_{60}$ given in Table II, the transition-temperatures on heating and cooling are quite sharp throughout the whole range. If the two paraffins, however, differ by two carbon atoms, then the transition-points on both heating and cooling between the limits of 30 and 70 % of one constituent are not sharp and occur gradually over a range that may be 0·5–1°. In the case of small amounts of impurities, such as 1–5 % of another paraffin, the sharpness of the transition-point is not affected. If however any of these paraffins are melted side by side in the bath with the pure paraffin there is always a recognisable difference in the temperature of transition on heating and especially on cooling.

A careful observation of the transition-point in fact will detect 1 % impurity in a paraffin. It is obvious that the exact temperature at which this transition occurs will depend a little on the rate of heating or cooling of the bath and therefore to make full use of this transition-point it is necessary to have a sample of chemically pure paraffin for comparison.

We have carefully compared the melting-points and transition-points of pure paraffins with those containing other paraffins in the small proportion of 1–5 % as given in Table V. The melting-points of the latter in all cases are raised or lowered according as to whether the admixed paraffin has a longer or shorter chain. When such samples are melted side by side the difference in melting-point is observable though it may be too small to be stated. However, the values obtained for transition-points, especially on cooling, differ appreciably and are never less than 0·2°.

In general our observations suggest that the even number paraffins containing 1–5 % of a neighbouring paraffin show considerably more depression in the transition-point on heating than do the odd number paraffins (Fig. 8).

In the case of approximately equimolar mixtures of two paraffins separated by three or more carbon atoms, the transition-point is not well defined. The contents of the melting-point tube viewed at room temperature show very little crystalline structure. A glassy state is already apparent, and on heating there is no definite first transition. The second transition is however very marked and occurs at 1–1·5° instead of 0·3–0·5° below the melting-point. On cooling the first transition to the glass takes place normally, and this form persists down to room temperature with, perhaps, the appearance of a few cleavages, showing partial reversion to the more usual crystalline form. As will be emphasised later, this marked difference in behaviour is of great practical use.

In the case of equimolar mixtures of three or four paraffins the transition-temperature is very indistinct and occurs at that of the lowest member in the mixture. The transition-point, therefore, can be 10–15° below the actual point of fusion. The one sample of an equimolar mixture of five paraffins that was made, $C_{26}H_{54}$ to $C_{30}H_{62}$ inclusive, showed no appreciable transition-points at all.

General discussion.

It will be seen from the experimental data given above that if a paraffin is to be considered pure it must have not only the correct melting-point, but also the correct transition-point. Its spacing will be correct to 0·2 % and the picture will show 8 or more orders. It will also give good microcrystals which display a sharp rhombic cleavage, the angles of which will differ according as to whether the paraffin has an odd or even number of carbon atoms.

In biochemical investigations it is improbable that paraffins of this high degree of purity will be often encountered. Recognising this fact we have, in this research, aimed not only at the establishment of these absolute criteria of purity, but also the collection of evidence which will enable us to suggest the composition of a mixture from which we are unable to separate a chemically pure paraffin. In the following summary we give a number of conclusions which are already aiding us in our investigations of plant waxes. In passing it may be mentioned that mixed paraffins can be readily separated into fractions having different melting-points by means of light petroleum, but that the preparation of a single paraffin of recognisable degree of purity from a mixture is always very laborious, and according to our experience, often impossible.

(1) If a paraffin gives the correct melting-point, and also the correct transition-temperature on both heating and cooling, then it contains less than 1 % of impurity. In such cases confirmation by X-ray analysis is unnecessary.

(2) X-ray analysis alone, without due regard to melting- and transition-points, is insufficient to identify a paraffin. Reference to Table III will show that $C_{31}H_{64}$ and a mixture of 20 % $C_{29}H_{60}$ + 80 % $C_{31}H_{64}$ both have the same spacing (41·5 Å.).

(3) The melting-point curve of a mixture of two paraffins which do not differ by more than 2 carbon atoms is a straight line (Fig. 9). This means that the melting-point of a mixture is determined by its mean molecular weight, and that no depression is exhibited. Within similar limits as to carbon atoms the same is true for mixtures of three paraffins. In respect to melting-point therefore the mixed paraffins are sharply differentiated from the alcohols and acids, which show in most cases very considerable depression. This finding has a very important practical application. In separating the constituent paraffins of a mixture it follows that of any two fractions obtained the one with the higher melting-point will have the higher mean molecular weight. This, of course, is not necessarily so with mixtures of acids or alcohols, in which both the higher and lower acids or alcohols may have melting-points higher than that of the respective mixtures. Francis, Piper and Malkin [1930, p. 227] came to the conclusion that it was extremely difficult, and in some cases impossible, to effect the separation of mixed acids by fractional crystallisation, and it is probable that this also applies to alcohols.

Further we have collected a very large number of observations on the crystallisation of mixed paraffins, and since their layer structure is simpler

than those of the acids and alcohols (which contain the length of two molecules between the 001 planes) the interpretation of the X-ray photographs, at this stage, is more straightforward. For this reason we are investigating the composition of the mixed primary alcohols which occur in plant waxes by fractionation of the derived paraffins, in preference to the fractionation of the alcohols themselves or the derived acids as done by previous workers.

(4) It follows from the data given in the above section that a mixture of two paraffins can give the same melting-point as a pure paraffin, but the transition-point will be lower. When such a mixture is melted with this pure paraffin the melting-point will be unchanged, but the transition-point will be either that of the mixture or lower. Therefore an observation confined to the mixed melt—which is the usual laboratory practice—is, by itself, quite insufficient to warrant any conclusion as to purity. The transition-temperature gives this information at once.

(5) If a mixture has a recognisable transition-point, a sharp melting-point, and sets crystalline when the temperature on cooling falls about 0·5°, it is safe to assume that there are not more than three components present in recognisable amounts. If the material melts over an extended range (0·5–1°), and if on cooling crystals come out at a temperature on or above the lower of these limits, then the mixture is probably of two paraffins only whose carbon contents differ by three or more carbon atoms.

(6) The short spacing of an even number paraffin will come up although the sample contains 2·5 % of a longer or up to 10 % of a shorter paraffin. A relatively impure sample of an even number paraffin will therefore show a short spacing which is quite specific and is a positive identification. Unfortunately no such simple means are available for the positive identification of an odd number paraffin of this degree of purity.

(7) In a series of fractions obtained from a mixed paraffin which does not appear to contain more than three components our experience suggests that if the transition-temperatures are some 8–10° below the melting-points, components are even number paraffins, and if 6–8° they are odd. This conclusion must be accepted with caution, as the transition-temperatures are susceptible to very small amounts of impurities.

(8) When attempting to decide upon the constituents of a naturally occurring paraffin in which the maximum degree of separation has been obtained, we first measure the spacing. We are then able to select one or more binary synthetic mixtures which have the same spacing. Of these synthetic mixtures we choose the one in which both the M.P. and transition-point are in best accord with those of the mixture under examination. It is unusual to find a perfect fit, but quite common to find reasonable agreement between the spacings and M.P. Transition-points are considerably depressed by the addition of a small amount of impurity, and it is usual to find the transition-point, when not agreeing with a synthetic mixture of corresponding M.P. and spacing, to be low. In such cases we consider the main constituents of the mixture under

examination to be those of the best agreeing binary mixture with a small (about 5 %) impurity whose constitution we are unable definitely to state. Since we are concerned with establishing the actual presence in bulk of the main constituents rather than in estimation of the exact proportion in which they occur, it is our practice to find the spacings of mixtures which we have not actually prepared from graphs shown to correspond with those of similar mixtures whose spacings have been measured. Thus the spacing curve for $C_{28}H_{58}$ and $C_{30}H_{62}$ is taken to be identical with that for $C_{26}H_{54}$ and $C_{28}H_{58}$, but the scale of ordinates is raised to lie between the appropriate spacings of $C_{28}H_{58}$ and $C_{30}H_{62}$. Similar estimations have been made for transition-points. In each case we have checked this assumption by making one or two appropriate mixtures.

It will be seen from the above recommendations that our experiments, which have been guided throughout by X-ray analysis, bring out quite clearly the importance of the transition-point. In our investigations of plant waxes we are, in fact, now able to dispense entirely with the laborious X-ray analysis in all cases except (*a*) for positive identification, especially with even number paraffins, (*b*) as a guide to the composition of mixtures from which we are unable to obtain fractions of the requisite purity by fractional crystallisation. In the two papers which follow we describe the fractionations of plant waxes which were carried out at the same time as the work described in the present paper. In these cases X-ray analysis was freely used and it will be seen that the final results give, in several instances, practical confirmation of the recommendations given above.

The position of side chains.

Two important papers by Shearer [1925] and Savile and Shearer [1925] have shown how the position of a side-group in a straight chain may be determined by X-rays. We have used this method for identifying ketones and secondary alcohols.

The method may be understood from an inspection of Plate VIII, Fig. 3, which shows the spacings of a series of nonacosanones. All the chain lengths are the same, and the spacings, within 0·25 %, agree with those for nonacosane. The intensities of the lines vary in a marked manner, displaying a periodic intensity variation superimposed on the normal decrease with increasing order. A group at $\frac{1}{3}$ of the chain length gives weak 3rd, 6th, 9th, *etc.*, orders; half-way along the chain it causes the even orders 2, 4, 6, *etc.*, to be very faint. We find that the secondary alcohols have intensity fluctuations of an identical nature, but that the spacings are very much shorter, showing that the molecule is inclined. Also they have both a high and a low temperature form. The spacings give the chain length, and the intensity fluctuations decide the position of the group within about 2 carbon atoms. It must be added that Shearer has pointed out that when the side group is on the last or the penultimate carbon atom the

Table VI. *Melting-points and crystal spacings of ketones and secondary alcohols.*

Abbreviated formula of compound	M.P.	Series number	Crystal spacing in Å.		Remarks
$C_{14}-CO-C_{14}$	80·8°	C 1	38·65		The constant spacing but variable intensity distribution are shown in Plate VIII, Fig. 3
$C_{13}-CO-C_{15}$	74·7°	C 47	38·9		
$C_{11}-CO-C_{17}$	74·7°	C 48	38·9		
C_9-CO-C_{19}	75·0°	C 49	38·9		
C_9-CO-C_{20}	74·4°	—	38·8		
			High temp.	Low temp.	
$C_{11}-CHOH-C_{11}$	75·2°	C 6	—	31·5	
$C_{13}-CHOH-C_{13}$	80·5°	—	34·75	36·6	
$C_{14}-CHOH-C_{14}$	84·0°	—	—	37·15	Spacing lower than for the corresponding ketone
$C_{13}-CHOH-C_{15}$	79·5°	—	—	—	Spacing not observed
$C_{11}-CHOH-C_{17}$	74·7°	—	—	36·9	
$C_9-CHOH-C_{19}$	75·0°	—	—	—	
d-$C_9-CHOH-C_{19}$	82·2°	C 75	—	38·65	Note the high value of this spacing. It corresponds to the A spacing of *n*-nonacosane
$C_9-CHOH-C_{20}$	75·8°	C 55	—	37·15	
$C_{15}-CHOH-C_{15}$	84·7°	—	39·4	41·25	

Table VII. *Melting-points and crystal spacings of primary alcohols.* Malkin [1930].

Number of C atoms	M.P. (Malkin)	M.P. (A.C.C.)	Crystal spacing in Å. High temperature	Crystal spacing in Å. Low temperature
16	49·0°	—	44·9	37·4
18	59·0°	—	50·2	41·35
19	62·0°	—	52·8	—
20	—	65–65·5°	—	46·2
21	68·5°	—	56·9	57·4
22	72·0°	—	—	49·95
24	76·5–77°	75·6–76°	—	54·0
25	78·5°	77·7–78°	—	68·5

The spacing and M.P. of normal eicosanol are new measurements.

spacing is doubled, owing to crystallisation occurring with 2 carbon chains between the 001 planes like end to like end.

The melting-points and crystal spacings of the secondary alcohols and ketones which appear to us of biochemical importance are given in Table VI. Equimolar mixtures in all cases show a depression of many degrees. We have to thank Dr Malkin for permission to reproduce his data for the higher primary alcohols given in Table VII.

Summary.

Highly pure paraffins from $C_{26}H_{54}$ to $C_{36}H_{74}$ and certain long-chain ketones and secondary alcohols of possible biochemical importance have been synthesised and their crystal spacings determined.

If the melting-point of a pure paraffin is determined in the way described it is possible to determine accurately two transition-temperatures at which a visible change of structure occurs. The lowest of these is markedly affected by impurities and is shown to be of great practical importance.

The melting-points, transition-points and crystal spacings of a comprehensive series of mixed paraffins have been determined.

From the data thus obtained it has been possible to draw up a series of recommendations which will aid in the fractionation and identification of the mixed paraffins which occur in nature, or which can be obtained from the mixed primary alcohols which occur in nature.

Brief reference is made to the work of Shearer [1925] showing how the position of a side group in a straight chain can be determined by X-rays.

In conclusion we should like to thank Prof. Garner and Dr Malkin for supplying us with samples of highly pure paraffins and alcohols, and the Imperial Chemical Industries, Ltd., for a grant towards the expenses of this research.

REFERENCES.

Backmann and Clark (1927). *J. Amer. Chem. Soc.* **49**, 2089.
Brooks (1922). The non-benzenoid hydrocarbons. (New York.)
Channon and Chibnall (1929). *Biochem. J.* **23**, 168.
Drew and Porter (1928). *J. Soc. Chem. Ind.* **47**, 17.
Francis, Piper and Malkin (1930). *Proc. Roy. Soc. Lond.* A **128**, 214.
Garner, van Bibber and King (1931). *J. Chem. Soc.* 1533.
Gascard (1921). *Ann. Chim.* (9), **15**, 332.
Gluud (1919). *Ber. deutsch. chem. Ges.* **52**, 1051.
Grün, Ulbrich and Krczil (1926). *Z. angew. Chem.* **39**, 421.
Hildebrand and Wachter (1929). *J. Amer. Chem. Soc.* **51**, 2487.
Kraft (1882). *Ber. deutsch. chem. Ges.* **15**, 1714.
Levene, West and Van der Scheer (1915). *J. Biol. Chem.* **20**, 521.
Malkin (1930). *J. Amer. Chem. Soc.* **52**, 3739.
Müller and Savile (1925). *J. Chem. Soc.* **127**, 602.
—— —— (1928). *Proc. Roy. Soc. Lond.* A **120**, 437.
—— —— (1930). *Proc. Roy. Soc. Lond.* A **127**, 417.
Peterson (1906). *Z. Electrochem.* **12**, 141.
Piper, Brown and Dyment (1925). *J. Chem. Soc.* **127**, 2194.
—— Malkin and Austin (1926). *J. Chem. Soc.* 2310.
—— and Malkin (1930). *Nature*, **126**, 278.
Pregl (1924). Quantitative organic analysis. Trans. Fyleman. (London.)
Robinson and Robinson (1925). *J. Chem. Soc.* **127**, 2194.
Savile and Shearer (1925). *J. Chem. Soc.* **127**, 592.
Shearer (1925). *Proc. Roy. Soc. Lond.* A **108**, 655.

CCXXVI. THE WAX CONSTITUENTS OF THE APPLE CUTICLE.

By ALBERT CHARLES CHIBNALL, STEPHEN HARVEY PIPER, ALFRED POLLARD, JAMES ANDREW BUCHAN SMITH and ERNEST FRANK WILLIAMS.

From the Biochemical Department, Imperial College of Science and Technology, South Kensington, and the Wills Physical Laboratory, University of Bristol.

(*Received October 31st, 1931.*)

Introduction.

In the introduction to the preceding paper dealing with the synthesis and crystal spacings of certain higher paraffins, alcohols and ketones, it was pointed out that the object of the work was to enable a more thorough investigation of the chemical constitution of various plant waxes, especially leaf waxes, to be made than had hitherto been possible. The leaf waxes to which it was hoped particular attention could be given were those derived from various forage grasses, and preliminary investigations showed that these differed from that found by Channon and Chibnall [1929] in the cabbage leaf in that they contained large amounts of alcohols instead of a paraffin and ketone and were therefore not immediately amenable to the separation used by these authors. As a preliminary to work on the grasses it was necessary to devote some time to experiments, the aim of which was to separate fairly completely the following classes of compounds, *viz.* paraffins, ketones, primary alcohols and secondary alcohols, and because a survey of the literature suggested that these might all occur in the wax obtained from apple peel, that material, which can readily be obtained in quantity, was used.

Before describing the actual scheme of separation it may perhaps be as well to state briefly the results of previous workers who have investigated this apple peel wax. Power and Chesnut [1920] isolated a substance, m.p. 63°, which appeared to be a hydrocarbon and which was identified from its melting-point as *n*-triacontane $C_{30}H_{62}$. A more comprehensive investigation was made by Sando [1923], who extracted the peel with ether and obtained two groups of substances, one insoluble in light petroleum with which we are not now concerned, and the other soluble in light petroleum, from which he was able to obtain, by intensive fractional crystallisation, the hydrocarbon previously identified by Power and Chesnut and an alcohol melting at 81·5 to 82°. This alcohol gave an acetate melting at 44–46° and was therefore thought to be different from ceryl alcohol, which gives an acetate, m.p. 64–65°. 14-Heptacosanol, previously synthesised by Kipping [1893] melted at 80·5–81·5°

and gave an acetate melting at 45–45·5°. On a similarity of melting-point and without any further evidence at all, Sando identified his apple alcohol with this secondary alcohol, a finding which has been accepted without question by Rivière and Pichard [1924]. Sando's results suggested that other products besides this alcohol and hydrocarbon were probably present in his apple material. Previous work carried out by Power and also in this laboratory suggested that these other products might be primary alcohols or ketones, and the wax, therefore, seemed to be an excellent raw material with which to experiment on methods to separate these four products.

A METHOD FOR THE SEPARATION OF THE COMPONENTS OF PLANT WAXES.

It has long been known that primary alcohols, such as ceryl and myricyl alcohol, form hydrogen phthalates which give sodium salts insoluble in water, and this property has been utilised by a number of workers, notably by Power and his colleagues, for the isolation of such alcohols from plant extracts and especially from essential oils. With a view to finding out the applicability of this reaction to the separation of primary alcohols from material that may contain paraffins, ketones and perhaps secondary alcohols, experiments were made to find out the optimum conditions for the conversion of the alcohol into its hydrogen phthalate.

Using ceryl alcohol (M.P. 79·7°) obtained from Chinese wax it was found that heating with an excess of phthalic anhydride with or without pyridine at 120° for 18 hours gave the half ester in almost theoretical yield. On shaking the ethereal solution with aqueous sodium carbonate the insoluble sodium salt was precipitated and could be separated by centrifuging. The alcohol could be recovered from this sodium salt or from the half ester in good yield by saponification with alkali. When a secondary alcohol, however, such as 14-heptacosanol, was submitted to similar treatment it was found that the hydrogen phthalate when shaken in ethereal solution with aqueous sodium carbonate gave no precipitate of insoluble sodium salt. The sodium salt, in fact, was found to be extremely soluble in both ether and alcohol and insoluble in water. This at once suggested a possible method of separating a secondary carbinol not only from primary alcohols but also from paraffins and ketones, and in practice it has been found to work extremely well.

The crude plant wax is first saponified to break down any true wax esters and the unsaponifiable material removed in the usual way. This unsaponifiable material is then heated for 18 hours with phthalic anhydride to convert all the alcohols present into hydrogen phthalates. Excess of phthalic anhydride is then removed and the mixed phthalates, together with any unchanged material, dissolved in ether. The ethereal solution is then gently shaken with a slightly warm aqueous solution of sodium carbonate. The sodium salts of the primary phthalates are precipitated and can be quantitatively separated by appropriate treatment. The ethereal solution which now contains the sodium salts of the secondary phthalates and perhaps ketones and paraffins is evaporated

to dryness and taken up in a relatively small volume of boiling alcohol. On cooling, the paraffins and ketones crystallise out quantitatively leaving in the mother-liquor the very soluble sodium salts of the secondary alcohol esters. The mixture of paraffins and ketones can then be separated by the method used by Channon and Chibnall [1929] in which the ketone is converted into its oxime and advantage is taken of the greater solubility of this oxime in solvents such as light petroleum and mixed ether-acetone. It will be seen that this relatively simple procedure separates at once the primary alcohols, the secondary alcohols, ketones and paraffins into four distinct fractions, each of which can be readily purified. In practice certain modifications have been found essential. For instance, a second treatment with phthalic anhydride for 18 hours is always necessary to ensure that all the alcohols have been converted into their hydrogen phthalates. Also recent experience has shown that it is advantageous to remove hydrocarbons—which are fairly readily soluble in cold light petroleum—before treatment with phthalic anhydride. The method and its modifications will be dealt with in greater detail in the practical part of this and succeeding papers.

The method of separation applied to apple peel wax.

Applied to the unsaponifiable portion of apple peel waxes (50 g.) this method of separation yielded without any difficulty a secondary alcohol, a mixture of primary alcohols and a mixture of paraffins but no ketones.

The secondary alcohol. The alcohol recovered after saponification of the ether- and alcohol-soluble sodium salts on purification melted at 81·9–82·2° and was obviously the product previously isolated by Sando [1923], for it gave an acetate melting at 44·5–45°. A mixed melt however with 14-heptacosanol was depressed to 70–71°, showing conclusively that Sando's guess was wrong. One of the reasons which prompted us initially to investigate apple peel wax was the belief that this alcohol might be 15-nonacosanol, the secondary alcohol corresponding with the ketone, 15-nonacosanone and the paraffin, *n*-nonacosane, isolated by Channon and Chibnall [1929] from the cabbage. Synthetic 15-nonacosanol, however, melted somewhat higher, 83·8–84°, and a mixed melt was depressed to 78·5–79·5°, showing that the alcohols were not identical. The carbinol had a spacing of 38·65 Å., indicating a straight chain of 29 carbon atoms. Furthermore, the intensity of the different orders of reflection showed that the polar group must be about one-third of the distance along the chain from one end because the third and sixth orders were missing. X-ray analysis, therefore, suggested that the alcohol must be either 9-nonacosanol or 10-nonacosanol. To verify this supposition it was necessary to synthesise both these alcohols *via* the corresponding ketones. Meanwhile, in order to meet the synthesis, the apple alcohol was oxidised by means of chromium trioxide in glacial acetic acid to the corresponding ketone. The apple ketone, M.P. 74·7–74·9°, showed no depression when mixed with synthetic 10-nonacosanone, M.P. 74·5–75°, and a depression of 5° when mixed with synthetic 9-nonacosa-

none, M.P. 73·6–74·3°. Furthermore, the crystal spacings (Series Nos. C 35, C 49) of the derived apple ketone and of the synthetic 10-nonacosanone were identical (38·9 Å.), both, as before, having the third and sixth orders missing. The identity of the ketone derived from the apple alcohol was therefore definitely established. On reducing the synthetic 10-nonacosanone to the corresponding carbinol, 10-nonacosanol, however, it was found to melt at 75–75·2°, *i.e.* 7° lower than the apple alcohol. A mixed M.P. (78–79°) was about midway between the melting-points of the two substances, whereas a mixed melt of the apple alcohol with synthetic 9-nonacosanol (M.P. 75·5–75·8°) was depressed to 70°. Clearly the apple alcohol was not identical with the synthetic 10-nonacosanol, although no doubt closely allied to it. This finding at once drew our attention to the fact that if the apple alcohol had the constitution which its derived ketone suggested, it would possess an asymmetric carbon atom and therefore would be optically active, whereas the alcohol obtained by reduction of the ketone would of course be inactive. Dr Hirst of the University of Birmingham kindly undertook to observe the rotation of the apple alcohol, and he reported as follows: "The substance was examined in solution in chloroform (*c*, 1 %). The rotation observed in a 4 dm. tube was certainly less than the experimental error due to the instrument (± 0·01°). Photographic examination over the range λ 7000–λ 3800 in a 2 dm. tube showed that throughout this range there was no observable rotation (limits of experimental error here were ± 0·02°). It is therefore most improbable that the substance is optically active either in chloroform or in any other solvent." There remained the possibility that the length of the two paraffin side-chains was so great that optical activity might be reduced below measurable limits. This hypothesis was confirmed when the ketone obtained from the apple alcohol by oxidation was reduced. The regenerated alcohol melted at 74·5–74·9° and gave no depression in M.P. with the synthetic product, which melted at the same temperature. Attempts to racemise the natural product by heating gave no change in melting-point. At a later stage of the research we were able to prepare some 4 g. of the hydrogen phthalate. A 20 % solution in chloroform gave a small but definite rotation $[\alpha]_{5461}^{17\cdot5} = +0\cdot62°$. The apple secondary alcohol is therefore undoubtedly *d*-10-nonacosanol and it belongs to a small group of optically active compounds, of which mannitol is a well known example, exhibiting no appreciable rotation. It is possibly identical with the alcohol, M.P. 82·5°, isolated by Kawamura [1928] from the fruit of *Ginkgo biloba* which gave an acetate M.P. 43–43·5° and a ketone M.P. 74°.

Primary alcohols. The ether-insoluble sodium salts of the primary phthalates were saponified and yielded, after purification by crystallisation, 4·1 g. of primary carbinol. This material melted at 81° and when submitted to an intensive fractional crystallisation yielded fractions differing in M.P. by 1 and 2°. Investigations of these fractions soon convinced us that we were dealing with a mixture of primary alcohols of the type usually referred to as ceryl alcohol, the constitution of which will be discussed in greater detail in the

following paper. An upper fraction, M.P. 81·7–82·2°, gave an analysis corresponding closely with *n*-octacosanol. (Found: C, 81·8; H, 14·1. $C_{28}H_{58}O$ requires C, 81·8; H, 14·2 %.) The crystal spacing (Series No. C. 56) was 77·6 Å. which suggests that the product, according to the data of Malkin [1930] was $C_{29}H_{60}O$. On reduction *via* the iodide it gave a paraffin M.P. 62·2–62·8°. The crystal spacing (Series No. C. 72) was 38·4 Å. with only 5 orders showing, and the paraffin was clearly a mixture. A lower fraction, M.P. 80·2–80·7°, gave a spacing (Series No. C. 33) of 76·1 Å. and a derived paraffin which melted at 60–60·2°. The crystal spacing (Series No. C. 46) of this was 37·4 Å. with only three orders showing and again the material was definitely a mixture. All the fractions of this primary alcohol therefore were united and reduced *via* the iodide to the corresponding paraffin, M.P. 61·0–61·5°, which was submitted to an intense fractionation by means of light petroleum. This was found to be very laborious. After about 100 fractions had been dealt with the upper and lower limits of M.P. were 62·8° and 59·5°, but the main bulk of material still melted around 61° and 62°. The highest and lowest fractions were again treated with sulphuric acid. At this stage of the resolution we found that all the fractions showed certain characteristics. (1) The M.P. was sharp, and about 0·2–0·3° above the S.P. (2) On setting, the molten material crystallised out in fine needles. (3) The transition changes were well marked, but the temperature range in some cases was 1–2°. This convinced us that we were dealing with a mixture containing not more than 3 components in recognisable amounts, but the resolution was not sufficiently complete for X-ray analysis to be of any use. A fraction (Series No. B. 80) of M.P. 61·4–61·6°, corresponding to $C_{28}H_{58}$, gave a spacing 38·3 Å. with a fair number of orders, a melted layer however gave a poor picture and a spacing of 37·8 Å. This distortion of a spacing on melting is characteristic of a mixture of at least three paraffins, and showed that it was necessary to carry the fractionation still further. After another 150 fractions had been dealt with we reached a stage when further fractionation by this means seemed impossible. We had then 22 samples. All the fractions set on cooling in large crystal masses, but in none was the transition-temperature as high as that of a corresponding pure paraffin. We drew the conclusion that the fractionation had separated the three components to such an extent that each sample now contained only two of these in recognisable amounts. Ten representative samples were then X-rayed, and as was expected, the picture obtained was sufficiently good to enable a comprehensive analysis of the mixtures to be obtained.

A review of the data in Table I shows that it is possible to account for all the melting- and transition-points and the spacings from suitably chosen mixtures of $C_{30}H_{62}$, $C_{28}H_{58}$ and $C_{26}H_{54}$. On this assumption the bulk of the material in B. 115 is the middle paraffin with about equal amounts of the higher- and lower-melting constituents as impurities. In B. 110–B. 114 we have mainly $C_{30}H_{62}$, $C_{28}H_{58}$; in B. 116–B. 119 $C_{28}H_{58}$, $C_{26}H_{54}$. The transition-temperatures are nearly all slightly lower than those of the suggested binary

Table I. *Paraffin fractions obtained from the mixed primary alcohols.*

Series No.	M.P.	S.P.	Transition-point on heating	Spacing Å.	Synthetic mixture: Series No.	Synthetic mixture: Composition	M.P.	S.P.	Transition-point on heating	Spacing Å.	Remarks
B. 110	63·2–63·6°	63·2°	54·5°	39·25	Computed data	60 % $C_{28}H_{58}$ 40 % $C_{30}H_{62}$	63·2°	—	54·8	39·25	The photograph of B. 110 is definitely that of a paraffin with 30–40 % contamination.
					C. 97	95 % $C_{29}H_{60}$ 5 % $C_{31}H_{64}$	63·6°	63·1°	57·2°	39·15	C. 97 gives very good pictures.
					Computed data	90 % $C_{29}H_{60}$ 10 % $C_{30}H_{62}$	64°	—	55·2°	39·25	
B. 112	63·1–63·3°	63·1°	54–55°	38·85	Computed data	65 % $C_{28}H_{58}$ 35 % $C_{30}H_{62}$	63°	—	55°	39	
						$C_{29}H_{60}$	63·4–63·6°	—	57·3°	38·68	A very small % of $C_{30}H_{62}$ or $C_{31}H_{64}$ in the $C_{29}H_{60}$ would give the correct spacing. The photograph however is definitely that of a paraffin with 30–40 % of contamination.
B. 113	62·6–62·8°	62·4°	53·7–54·2°	38·55	Computed data	75 % $C_{28}H_{58}$ 25 % $C_{30}H_{62}$	62·5°	—	54·8°	38·5	
					B. 89	50 % $C_{29}H_{60}$ 50 % $C_{28}H_{58}$	62·3–62·6°	62·3°	55·6°	38·5	
					Computed data	35 % $C_{27}H_{56}$ 65 % $C_{29}H_{60}$	62°	—	54°	38·5	
					B. 122	34 % $C_{26}H_{54}$ 66 % $C_{29}H_{60}$	60·7–61°	60·7°	50–52°	38·6	
B. 114	62·2–62·6°	62°	53·2–53·7°	38·55	B. 89	50 % $C_{29}H_{60}$ 50 % $C_{28}H_{58}$	62·3–62·6°	62·3°	55·6°	38·5	
					Computed data	75 % $C_{28}H_{58}$ 25 % $C_{30}H_{62}$	62·5°	—	54·8°	38·5	
					B. 125	74 % $C_{28}H_{58}$ 24 % $C_{29}H_{60}$ 2 % $C_{26}H_{54}$	62·3–62·5°	62·0°	53–53·5°	38·5	

Series No.	M.P.	S.P.	Transition-point on heating	Spacing Å.	Synthetic mixture: Series No.	Synthetic mixture: Composition	M.P.	S.P.	Transition-point on heating	Spacing Å.	Remarks
B. 115	61·2–61·5°	61·2°	52–52·7°	38	Computed data	85 % $C_{28}H_{58}$ 15 % $C_{30}H_{62}$	62·1°	—	54·8°	38	
					C. 114	$C_{28}H_{58}$	61·4–61·5°	61·1°	57–57·4°	37 33·25	There was no appearance of the shorter spacing in B. 115.
					B. 123	50 % $C_{27}H_{56}$ 50 % $C_{29}H_{60}$	61·1–61·3°	60·9°	52–52·5°	38·2	
					B. 70	**90 % $C_{28}H_{58}$** 5 % $C_{26}H_{54}$ 5 % $C_{30}H_{62}$	**61·3–61·5°**	**61·2°**	**54–54·4°**	**38**	Appears to be the most probable constitution.
					B. 69	90 % $C_{28}H_{58}$ 5 % $C_{27}H_{56}$ 5 % $C_{29}H_{60}$	61·3–61·5°	61·2°	55°	37·7	
B. 116	59·9–60·1°	59·8°	50–51°	37·65	B. 47	**70 % $C_{28}H_{58}$** **30 % $C_{26}H_{54}$**	**60·1–60·2°**	**60°**	**52°**	**37·7**	
B. 117	58·0–58·4°	58°	47·2–47·4°	36·65	B. 46	**60 % $C_{28}H_{54}$** **40 % $C_{28}H_{58}$**	**58·6°**	**58·2°**	**49·5°**	**36·8**	
					B. 58	90 % $C_{27}H_{54}$ 10 % $C_{28}H_{56}$	59·2–59·5°	58·9°	52·5–53°	36·6	
B. 118	57·2–57·6°	57·1°	46–46·5°	36·24	B. 44	**70 % $C_{26}H_{54}$** **30 % $C_{28}H_{58}$**	**58°**	**57·5°**	**49·5°**	**36·2**	
B. 119	56·8–57°	56·6°	46·5–47°	35·55	B. 42	90 % $C_{26}H_{54}$ 10 % $C_{28}H_{58}$	57°	56·3°	49·5°	35·25	
					Computed data	55 % $C_{26}H_{54}$ 45 % $C_{27}H_{56}$	57·7°	—	49·8°	35·6	
					B. 124	**88 % $C_{26}H_{54}$** **9 % $C_{28}H_{58}$** **3 % $C_{30}H_{62}$**	**56·8–57°**	**56·6°**	**47°**	**35·51**	

The most probable constitution of each fraction is given in heavy type.

mixture, probably owing to the presence of traces of the lower paraffin in the top, and the higher in the lower fractions. We have verified this by making appropriate mixtures containing small amounts of the suggested impurity, as shown in the case of B. 114 and B. 119. There is no change in the M.P., S.P. or crystal spacing, but the transition-point is depressed about 2°. We have in each case tested the possibility of "odd + even" and "2 odd" paraffin combinations. While in some cases agreement is fair, in others it is necessary to assume only small amounts of one component, and the X-ray pictures definitely reject such a mixture. The behaviour of the material after partial fractionation, as mentioned above, suggests that it does not contain more than three components in any appreciable amount, and the three even number paraffins suggested above are the only ones that give consistent results throughout the series. Observations on the second transition-points prove that the chains do not differ by three or more carbon atoms, also if any one of the fractions is in the main composed of paraffins differing by four carbon atoms, the photographs would have shown the two sets of spacings. We consider that the above results show conclusively that the mixed primary alcohol consisted of *n*-triacontanol, *n*-octacosanol and *n*-hexacosanol.

The paraffin fraction. The crude fraction, recrystallised from carbon disulphide, melted at 63–64°. The product was slightly brown and was therefore dissolved in boiling alcohol and clarified with charcoal. The resulting product melted at 63·2–63·6°, and set at 62·4° with no definite crystal form. The material, in fact, reminded us of the crude hydrocarbon fraction of cabbage from which the ketone had not yet been separated. A portion of the product was therefore oximated and an attempt made to separate a ketoxime soluble in light petroleum, but none was found. Furthermore analysis showed that no large amount of a ketone or other oxygen-containing substance could be present in the mixture. The material was therefore repeatedly treated with sulphuric acid at 130° until no further blackening occurred. Contrary to our expectations the M.P. was not raised but was lowered to 62·6–63°. The crystal spacing was 38·7 Å. (Series No. C. 29). This spacing at the time seemed quite correct for $C_{29}H_{60}$. The M.P., moreover, agreed with that of the same material isolated by Channon and Chibnall [1929] from cabbage. But synthetic $C_{29}H_{60}$ melted at 63·4–63·6° and no amount of purification with sulphuric acid enabled us to raise the M.P. of the apple product above 63°. We were therefore forced to the conclusion that this apple product must be a mixture and in spite of this was giving a correct crystal spacing for a pure paraffin. This led us to the investigation of the crystal spacing of mixed paraffins and we then found that the spacing, for example 38·7 Å., of a pure paraffin could also be the spacing given by a mixture, as has been shown in the preceding paper [1931] dealing with these paraffins and their mixtures. We therefore attempted to fractionate the product derived from the apple and found that this could be slowly but effectively accomplished by making use of the different solubilities of the paraffins in cold petroleum (B.P. 40–60°). The fractionation, practical

details of which are given later, finally gave us a series of products whose melting-points and crystal spacings are given in Table II. The highest melting fraction, 63·4–63·6°, was further fractionated by means of petroleum (B.P. 40–60°) into three samples (C. 133–135). From the discussion which we gave in the preceding paper on the criteria necessary for judging the purity of a paraffin we consider that their melting- and transition-points and crystal spacings are definite proof that a highly pure sample of $C_{29}H_{60}$ has been isolated. When the data given in Table II are viewed as a whole it is seen that the variation in the crystal spacing with the melting-point is very similar to that which would be given by a mixture of $C_{29}H_{60}$ and $C_{27}H_{56}$. In the sixth column of Table II we have given the percentage composition calculated from the M.P. of a mixture of $C_{29}H_{60}$ and $C_{27}H_{56}$ and in column 7 the crystal spacing of such a mixture calculated from the synthetic mixture of $C_{31}H_{64}$ and $C_{29}H_{60}$ given in the previous reference. It will be observed that the suggested and observed crystal spacings are in very close agreement. We would call particular attention to fraction C. 138. Its melting-point suggests that it could be either $C_{28}H_{58}$, equimolar $C_{27}H_{56} + C_{29}H_{60}$ or 66 % $C_{29}H_{60} + 34$ % $C_{26}H_{54}$. A comparison of the crystal spacings and the transition-points shows clearly that it is equimolar $C_{27}H_{56} + C_{29}H_{60}$. Moreover we did not find, during the course of the fractionation, any product melting between 61·4–61·6° which could not be readily separated into a series of fractions having M.P. both higher and lower. We therefore draw the definite conclusion that the mixed paraffin contains only $C_{29}H_{60}$ and $C_{27}H_{56}$ and that $C_{28}H_{58}$ and $C_{26}H_{54}$ are absent.

General discussion.

Summarising the above results it will be seen that we have been able to prove definitely the presence in the apple peel wax of the following substances, *n*-nonacosane, *n*-heptacosane, *d*-10-nonacosanol, *n*-triacontanol, *n*-octacosanol and *n*-hexacosanol.

It is interesting to consider these results in relation to the hypothesis advanced by Channon and Chibnall [1929] on the metabolism of paraffins in the plant. If the paraffin (III) is synthesised from the fatty acids (I) *via* the ketone (II), then the discovery of a secondary alcohol such as *d*-10-nonacosanol together with *n*-nonacosane in the same wax is interesting. Both could, of course, be formed by reduction of the corresponding ketone, yet the reduction of —CHOH— to —CH_2— is so difficult, both chemically and by the aid of living organisms, that one cannot suggest that the alcohol is an

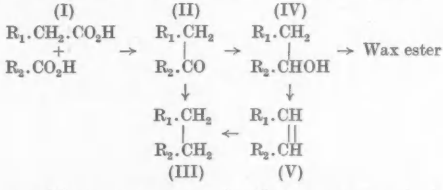


Table II. *Melting-points and crystal spacings of the paraffin fractions (with certain standards for comparison).*

Series No.	Transition-point on heating	Melting-point	Setting-point	Transition-point on cooling	Suggested composition %		Crystal spacing	
					$C_{27}H_{56}$	$C_{29}H_{60}$	Observed Å.	Calculated from Col. 6 Å.
C. 133	57·2–57·5°	63·4–63·6°	63·2°	55·8°	0	100	38·8	38·7
C. 134	57·5–57·8°	63·4–63·6°	63·2°	56·0°	0	100	38·6	38·7
C. 135	57–57·3°	63·4–63·6°	63·2°	55·3°	0	100	38·75	38·7
C. 136	55·2–55·8°	62·7–62·9°	62·3°	54·7°	17	83	38·85	38·8
C. 137	53·5–54°	61·5–61·8°	61·4°	52°	40	60	38·65	38·5
C. 138	52–52·5°	61·4–61·6°	61·2°	51·2°	44	56	38·35	38·4
C. 139	52°	60·5–60·7°	59·6°	50·4°	62	38	38·0	38·1
C. 140	51°	59·8–60·2°	58·8°	48·5°	76	24	37·4	37·4
					Composition of standards			
C. 26	57·5°	63·4–63·6°	63·2°	56·0°	$C_{29}H_{60}$		36·68	
C. 114	57·2–57·4°	61·2–61·5°	61·1°	53·7°	$C_{28}H_{58}$		37·0	
							33·25	
C. 22	52·8–53°	59·0–59·1°	56·5°	51°	$C_{27}H_{56}$		36·4	
B. 122	50–52°	60·7–61°	60·7°	Indistinct	66 % $C_{29}H_{60}$ 34 % $C_{28}H_{54}$		38·6	
B. 123	Indistinct 52–52·5°	61·1–61·3°	60·9°	51–50·5°	Equimolar $C_{27}H_{56}+C_{29}H_{60}$		38·2	

intermediary product in the direct reduction of the ketone to the paraffin. If the alcohol and paraffin have a common origin, and the fact that they both contain 29 carbon atoms is strong evidence on this point, then one would suggest (1) that the secondary alcohol had been formed by partial reduction of the ketone and that it is an alternative end-product to the hydrocarbon. It might, of course, unite with a long-chain fatty acid to form a true wax. (2) That the ketone (II) undergoes a reduction first to the secondary alcohol (IV) and then to the corresponding paraffin (III) *via* the corresponding olefine (V). Such a reduction involving the loss of water and the creation of a double bond is well known in biological reactions (*cf.* the interconversion of malic and succinic acids *via* the unsaturated fumaric acid). It is true that in the present case the ketone has not been found, but support for the hypothesis that it is the precursor of both the secondary alcohol and paraffin is obtained from a later research, in which it is shown that 15-nonacosanol, 15-nonacosanone and *n*-nonacosane are all present in the wax of the Brussels Sprout leaf. Our own researches have not yet given any definite evidence for the presence of olefines in plant waxes. There are, however, a few references in the literature to the isolation of products that appear to contain only carbon and hydrogen in the proportion close to that found in the paraffins and which have been stated to possess small iodine values. It is possible therefore that olefines corresponding with these ketones, alcohols and paraffins are present in the plant waxes.

Channon and Chibnall [1929] when they first put forward the hypothesis that the ketone might arise through the condensation of two molecules of fatty acids drew attention to the fact that the ketone they found in the cabbage leaf, 15-nonacosanone would postulate the presence in the plant at some stage in the synthesis of pentadecoic acid, and because the presence of this acid in natural products has not been clearly demonstrated, they considered that the hypothesis should be accepted with great caution. In the present instance the two fatty acids required to give the postulated intermediate ketone would be decoic and eicosanic acids, both of which have been found in plants, although neither of them can be considered a commonly occurring fatty acid. It will be seen then that the discovery of this secondary alcohol in the apple wax does, to a certain extent, support the original contention that these long-chain products can arise from the shorter fatty acids.

We still feel however that caution is necessary. We believe that for the first time some insight is being gained into the constitution of the mixed primary alcohols which we ourselves invariably find in these plant waxes. We cannot lose sight of the possibility that these primary alcohols are as closely connected with the paraffins as are the secondary products. If so, then we should expect to find some general relationships between the number of carbon atoms in the paraffins and in the primary alcohols. We have as yet very few data concerning this point, and further discussion at the present time does not seem profitable. Our chief difficulty in collecting such data lies in the fact that those waxes which contain a large proportion of prim alcohol

(*e.g.* Chinese wax, raphia wax) do not contain paraffins or secondary products, and those which contain paraffins (*e.g.* Candalilla wax) have only a small amount of primary alcohols. From the point of view of paraffin metabolism it is essential that all these be found in one and the same wax, and it is for that reason that we have thought it worth while to resolve, with much labour, the very small amount of mixed primary alcohol (5 g.) which we isolated from the apple peel.

Experimental.

Extraction of the wax. Messrs Lyons kindly provided us with a large quantity of apple peel which had been dried *in vacuo* at 40°. It was powdered in a coffee mill and then extracted with petroleum (B.P. 40–60°) in a Soxhlet for 15 hours. The extract from 8 kg. of peel was concentrated to 500 cc. and treated with two volumes of acetone. The precipitated wax was filtered off, again dissolved in the same volume of petroleum and precipitated as before. Dried *in vacuo* the crude wax (57 g.) was a yellow-brown, brittle solid of no definite M.P.

Saponification of the wax. An alcoholic solution of potassium hydroxide (170 cc. of 4·5 %) was added to the wax (57 g.) dissolved in warm benzene (300 cc.) and the mixture boiled for 3 hours on the water-bath. Water (300 cc.) was added and the mixture again heated for 10 minutes. The benzene layer was then separated, the aqueous solution extracted with benzene and the benzene solutions combined. On removal of the solvent by distillation the unsaponifiable matter was obtained as a pale brown hard wax (50 g.) M.P. 63·5°.

Treatment with phthalic anhydride. The unsaponifiable material (50 g.) was heated with phthalic anhydride (50 g.) and pyridine (20 cc.) for 18 hours at 120° in an oil-bath. In order to remove the pyridine the hot molten mixture was then poured into dilute hydrochloric acid and stirred while the mixture was heated to boiling-point. After cooling, the solid layer of phthalates was separated and heated with successive quantities of water to remove the residual phthalic anhydride. The mixture was allowed to cool and the solid cake of crude esters was collected and dissolved in warm ether (1500 cc.), giving a light brown solution. This was cautiously agitated with an excess of slightly warm aqueous solution of sodium carbonate, which caused the separation in the aqueous phase of the insoluble white sodium salts of the primary phthalic esters. The lower layer, which was an emulsion of water, ether and suspended solid matter, was separated from the supernatant ether and centrifuged. By this means the solid sodium salts collected at the ether-water interface and could be readily removed. This semi-solid mass was freed from most of the ether-soluble matter with which it was contaminated by shaking with a large volume of ether and centrifuging the resultant emulsion. The solid was then dried *in vacuo* and repeatedly extracted with dry ether. There remained a grey powder (12 g.) of no definite M.P. Many experiments on the separation of these primary phthalates have shown that the precipitated sodium salts are more granular and less gelatinous if the sodium carbonate solution is slightly warm.

Even so they are difficult to filter and can only be effectively separated by centrifuging.

After separation of these insoluble sodium salts the ether solutions were combined and the solvent removed. The residue was taken up in boiling alcohol and on cooling an impure paraffin fraction (32·2 g.) crystallised out. The mother-liquor on removal of the solvent yielded 7·2 g. of a gum which was readily soluble in cold ether, alcohol and most organic solvents, and which was the sodium salt of the secondary alcohol phthalate. The crude paraffin fraction (32·2 g.) was again heated with phthalic anhydride (15 g.)—this time without pyridine—for 18 hours at 120° and then treated as before. On shaking the ethereal solution with aqueous sodium carbonate, a slight precipitate of insoluble matter was formed. This was removed and added to the sodium salts of the primary phthalates. The ether solution was evaporated and the residue dissolved in a mixture of benzene (400 cc.) and alcohol (1600 cc.). A crystalline mass of the paraffin (23 g.) separated on cooling and on evaporation the mother-liquor yielded a further quantity (6 g.) of gummy substance which was also found to be the sodium salt of the secondary alcohol ester.

Primary alcohols. The sodium salts of the primary phthalates (12 g.) were hydrolysed by heating with a solution of potassium hydroxide (20 g.) in 95 % alcohol (100 cc.) for 18 hours. The hot solution was filtered and the crystalline mass which separated on cooling was filtered off and dried *in vacuo*. This material was then extracted many times with boiling ether, the ethereal solution filtered, and the solvent removed by distillation. The residue was taken up in petroleum (400 cc. B.P. 40–60°) and boiled with charcoal. From the clarified solution the primary alcohols separated as a white crystalline product (5·7 g.). On repeated crystallisation from various solvents, fractions, varying in M.P. from 80·7° to 82·2°, were obtained together with a small amount (0·9 g.) of crude paraffin, M.P. 62°. The discussion showing that these fractions represented mixed alcohols has already been given, p. 2099.

To obtain further evidence as to the constituent alcohols present in these mixtures they were all united and the combined material (5·0 g.) reduced *via* the iodide, to the corresponding paraffin (4·0 g.) M.P. 61–61·5°. This material was submitted to a prolonged fractionation by means of light petroleum (B.P. 40–60°) in the manner described later for the actual apple paraffin. About 250 samples were obtained during this operation, and there were finally obtained 22 samples weighing in all 1·5 g., whose melting-points and crystal spacings are given in Table I and have been fully discussed in the Introduction.

Secondary alcohols. The crude sodium salt (7·2 g.) from the first treatment with phthalic anhydride was heated with potassium hydroxide (10 g.) in alcohol (75 cc.) for 18 hours. The hot solution was filtered and the crystalline solid which separated on cooling was collected and recrystallised from petroleum (B.P. 40–60°). The product, a white crystalline solid (2·4 g.) melted at 80·7°, while the mother-liquor yielded 0·6 g. of material, M.P. 64°, which was added to the crude hydrocarbon fraction described later. The sodium salt (6 g.)

from the second phthalic anhydride treatment was saponified in the same way, and gave a white crystalline substance (2·9 g.), M.P. 80·9–81·2°, together with an impure fraction (0·65 g.) which was added to the crude paraffin fraction. The prolonged treatment with alcoholic potassium hydroxide which we used in this work to saponify the phthalates of the primary and secondary alcohols caused considerable decomposition and darkening of the solution. More recently an alternative method of hydrolysis with sodium ethoxide has been employed which gives a much better recovery of the alcohol. The ester (1 g.) dissolved in benzene (20 cc.) is heated to boiling with alcohol (10 cc.) in which sodium (0·7 g.) has been dissolved. A precipitate of sodium phthalate readily forms and after heating for 5 minutes the solution is kept for 2 hours. Water is then added and the benzene layer evaporated to dryness. Extraction of the residue by petroleum (B.P. < 40°) yields the carbinol as a white crystalline powder (0·7 g.). The two high-melting products from the soluble sodium salts mentioned above gave no depression in M.P. when mixed and appeared to be identical. To make absolutely certain that they were not still contaminated with primary alcohol they were mixed and again put through the phthalate treatment. A small precipitate of insoluble sodium primary alcohol esters was obtained. The secondary alcohol recovered by saponification (3·7 g.), M.P. 80·5–81·5°, was recrystallised from carbon disulphide, acetone and light petroleum, and was finally obtained in long white needles, M.P. 81·9–82·2°. The identification of this alcohol as *d*-10-nonacosanol has been described in some detail in the Introduction. (Found: C, 82·0; H, 14·3. $C_{29}H_{60}O$ requires C, 82·0; H, 14·2 %.) The mother-liquors after this fractionation were collected and the residue from them crystallised from a small amount of hot acetone. The material (1·25 g.), M.P. 73°, was again fractionated. It yielded 0·46 g. of alcohol, M.P. 79°, which was an impure specimen of *d*-10-nonacosanol and a series of paraffin fractions which melted between 60 and 67°. No evidence for the presence of any other secondary alcohol, for instance one with 27 carbon atoms corresponding to *n*-heptacosane, was found.

The hydrogen phthalate of the alcohol (4·85 g.) after purification was recrystallised from alcohol (charcoal) and was obtained as a white crystalline powder (5·25 g.), M.P. 54·5–54·7°. (Found: C, 77·1; H, 11·3. $C_{37}H_{65}O_4$ requires C, 77·4; H, 11·4 %.) For a 20 % solution in chloroform the observed rotation was $\alpha_{5461}^{17·5} + 0·25°$, whence $[\alpha]_{5461}^{17·5} = + 0·62°$.

d-10-Nonacosanol acetate was prepared in the usual way: M.P. 44·5–45°.

Paraffin fraction. The crude material was crystallised from benzene-alcohol (23 g.), M.P. 62·8–63·2°. (Found: C, 85·1; H, 14·7. $C_{29}H_{60}$ requires C, 85·2; H, 14·8 %.) The attempt in previous batches to obtain a pure paraffin from such a product by treatment with sulphuric acid has been described in the Introduction. To fractionate the constituent paraffins the material was stirred for 2–3 minutes at room temperature with petroleum (B.P. 40–60°) in the proportion of 10 cc. of solvent per g. of material. The solvent was filtered off, reduced in volume, and treated with warm acetone (2 : 1). The paraffin

crystallised out and was removed by filtration. This process was repeated until no more of the original material appeared to go readily into the cold solvent. In the present case there was a residue (3·7 g.) from which we were able to obtain no definite substance. Until the possible effect of saponification and treatment with phthalic anhydride on olefines has been investigated we are not examining this material further. The many fractions of paraffins obtained in this way showed a gradual rise in M.P. Each fraction was then further fractionated as follows. The material was completely dissolved by stirring at room temperature in 100 volumes of petroleum (B.P. 40–60°). The solution was then placed in an ice-chest. As it cooled the paraffin crystallised out and by using a cooled suction funnel it was possible to obtain about six successive crops of material. These were found to show a successive decrease in M.P. By dovetailing these two methods of fractionation there was obtained fairly readily a series of 10 fractions melting between 59 and 64°. These were then treated with sulphuric acid at 130° until no further charring occurred. The upper and lower fractions showed considerable charring, the middle ones practically none at all. After this treatment the products were again fractionated and there was finally obtained after some 200 fractionations in all a series of products whose melting-points and crystal spacings (Table I) have been discussed fully in the Introduction.

Fatty acids. The amount of fatty acids obtained on saponification of the original crude wax was very small. On decomposition of the sodium salts in the usual way after complete extraction of the unsaponifiable material there were obtained some 5 g. of crude fatty acid. The material was deep green and was obviously very impure, and to obtain a colourless product it had to be boiled many times with alcohol and charcoal. By fractional crystallisation there were then obtained (1) 0·65 g. of fatty acid, M.P. 82·2–82·8°, mol. wt. by titration, 470, clearly the usual mixture of high fatty acids of mean carbon content 28–30 found in many plant waxes, and (2) 2 g. of white crystalline fatty acid, M.P. 55°. These fatty acids may have been present as wax esters with long-chain alcohols or they may have been derived from the residual amount of glycerides present in the crude wax. The amount found, however, shows clearly that the major part of the alcohol present in the wax is free. In some earlier experiments the crude wax was fractionated by means of hot acetone. From a batch of 10 g. there was obtained in one case, 0·3 g. of material almost insoluble in boiling acetone which could be recrystallised from hot ethyl acetate. It melted at 79–80° and was clearly a true wax corresponding with the long-chain primary alcohol and the higher fatty acids which were afterwards isolated. (Found: C, 82·4; H, 13·8. $C_{28}H_{57}O.OC.C_{27}H_{54}$ requires C, 82·4; H, 13·7 %.)

SUMMARY.

The wax fraction of apple peel has been used to elaborate methods for separating the paraffins, ketones, primary alcohols and secondary alcohols which occur in many plant waxes.

The method of separation adopted is based on the solubility in ether and alcohol of the sodium salts of the secondary alcohol phthalates.

The following constituents of apple peel wax have been definitely identified. *n*-nonacosane, *n*-heptacosane, *d*-10-nonacosanol, *n*-hexacosanol, *n*-octacosanol and *n*-triacontanol.

The bearing of these findings on the metabolism of paraffins in the plant is discussed.

The preliminary work in this investigation was carried out at University College, London, and we wish to record our thanks to Prof. J. C. Drummond for the hospitality of his laboratory. We should also like to thank the Imperial Chemical Industries for a grant towards the expenses of this research, Dr L. H. Lampitt of Messrs Lyons for placing at our disposal large quantities of specially dried apple peel, and both Dr Hirst and Dr Anderson for examinations of optical activity.

REFERENCES.

Channon and Chibnall (1929). *Biochem. J.* **23**, 168.
Kawamura (1928). *Jap. J. Chem.* **3**, 89.
Kipping (1893). *J. Chem. Soc.* **63**, 452.
Malkin (1930). *J. Amer. Chem. Soc.* **52**, 3739.
Power and Chesnut (1920). *J. Amer. Chem. Soc.* **42**, 1509.
Piper, Chibnall, Hopkins, Pollard, Smith and Williams (1931). *Biochem. J.* **25**, 2072.
Rivière and Pichard (1924). *Compt. Rend. Acad. Sci.* **179**, 775.
Sando (1923). *J. Biol. Chem.* **56**, 457.

CCXXVII. THE WAX CONSTITUENTS OF FORAGE GRASSES.

I. COCKSFOOT AND PERENNIAL RYEGRASS.

By ALFRED POLLARD, ALBERT CHARLES CHIBNALL and STEPHEN HARVEY PIPER.

From the Biochemical Department, Imperial College of Science and Technology, South Kensington, and the Wills Physical Laboratory, University of Bristol.

(*Received October 31st, 1931.*)

Introduction.

In the present paper an account is given of an investigation into the wax constituents of two very commonly occurring foliage grasses, cocksfoot (*Dactylis glomerata*) and perennial ryegrass (*Lolium perenne*). It has not been found possible to obtain as complete an analysis as that recorded in the previous paper dealing with apple-peel wax, but the results are of great interest because they have enabled us to investigate the constitution of a long-chain primary alcohol which is the main constituent of these two waxes.

This alcohol has been shown to be a fairly pure sample of *n*-hexacosanol. Although this substance has been assumed to be present in many natural waxes and given the name of ceryl alcohol, evidence is produced that all the samples so far recorded in the literature are mixtures which may or may not contain *n*-hexacosanol.

No paraffin was found in the cocksfoot, although a small amount was present in the ryegrass. This had M.P. 66°. The literature contains many references to paraffins melting at this temperature, and from a similarity of melting-point they were always considered to be *n*-triacontane $C_{30}H_{62}$. We shall discuss paraffins of this type more fully in a later paper dealing with many samples of paraffins isolated by previous workers, who have very kindly placed samples of their products at our disposal for further analysis. In the present case it was quite easy to show that the paraffin, M.P. 66°, was a mixture.

It is shown in the discussion later that the hippocoprosterol of Bondzynski and von Humnicki [1896] and also of Dorée and Gardner [1908] is the so-called ceryl alcohol. The latter authors also show quite conclusively by feeding experiments that hippocoprosterol passes through the intestines of herbivorous animals unchanged. One can conclude therefore that the *n*-hexacosanol found in cocksfoot and perennial ryegrass, which may be present to the extent of 1 % of the dry weight of the grass, has no feeding value.

Experimental.

Cocksfoot (Dactylis glomerata).

Preparation of leaf extract. This was prepared by a modification of the method of Chibnall and Channon [1927]. The grass (fresh weight 33 kg., dry weight 4·9 kg.) was minced with water and the green colloidal solution separated from the cell-wall material and heated to give the "coagulum," which, after pressing, was first extracted in a Soxhlet apparatus with ether and then four times successively with boiling alcohol. The residue of leaf-cell material was pressed in a Buchner press at 500 kg./cm.^{2} until no more liquid exuded. This hard press-cake was then disintegrated in a coffee-mill and extracted first with ether and then with four successive portions of boiling alcohol. All the alcoholic extracts were united, the alcohol removed by distillation *in vacuo* and the gummy residue taken up in warm ether. The cooled ethereal solution was filtered and shaken three or four times in a funnel with water. This caused the separation of much material which coloured the aqueous layer deep brown. Emulsions were also formed which were difficult to break. The final ethereal solution was then added to the two ethereal extracts mentioned above and the whole taken to dryness; weight 277 g.

Preparation of the wax. The ethereal extract was dissolved in about 1000 cc. of warm ether and treated with two volumes of acetone. The precipitated wax and phosphatide were filtered off, redissolved in a similar quantity of warm ether and again precipitated with two volumes of acetone. The resulting product was then extracted some eight times with hot acetone, each acetone extract being filtered hot. On cooling, the crude wax crystallised out and was filtered off; weight 59·6 g. (equivalent to 21·7 % of the ethereal extract and 1·2 % of the dry weight of the grass taken).

Saponification of the wax. The wax (59·6 g.) was dissolved in warm benzene (500 cc.), a 10 % alcoholic solution of potassium hydroxide (300 cc.) added and the mixture boiled for 2½ hours on a water-bath. Warm water (700 cc.) was then added, the benzene solution separated in a funnel and the aqueous layer extracted several times with benzene. Emulsions formed during the extraction were readily broken by centrifuging. The aqueous alkaline solution after acidification gave only a trace of fatty acids. The benzene solution on evaporation gave a dark green viscous mass. To separate the unsaponifiable material from the potassium soaps of higher fatty acids the whole was extracted many times with boiling acetone, each extract being filtered hot. On cooling the unsaponifiable portion crystallised out and was removed by filtration. It was a hard greenish-yellow wax (45 g.).

Fatty acids. After extraction with hot acetone there remained a dark green residue. This material was heated with a small quantity of concentrated HCl, washed and again extracted with hot acetone. The hot filtered extracts deposited on cooling a dark green powder (7·75 g.) which appeared to be a mixture of higher fatty acids and the degradation products of chlorophyll. The

mother-liquors gave a residue of crude fatty acids (4·5 g.) which on extraction with petroleum (B.P. < 40°) gave a pale brown semi-solid mass of fatty acid (3·6 g.) similar to that given by saponification of the glyceride fraction. The dark green powder mentioned above was extracted in a Soxhlet apparatus with petroleum (B.P. < 40°). The extract yielded a pale brown substance (3·1 g.), M.P. 73–73·5°. This was saponified by heating with sodium ethoxide (0·5 g. sodium in 20 cc. of alcohol) and benzene (30 cc.) for 2½ hours. The benzene solution was separated, the solvent removed and the residue taken up in acetone. The acetone gave on evaporation a brown residue 0·15 g. The substance undissolved by the acetone was acidified with hydrochloric acid. It was now completely soluble in hot acetone. On cooling a pale yellow granular crystalline material separated (1·9 g.), M.P. 78·2–78·7°. Further purification yielded a white crystalline granular powder, M.P. 79·4–79·8°, mol. wt. by titration 440; $C_{29}H_{58}O_2$ requires 435. It was clearly the usual "cerotic acid" of plant extracts, which was shown to be a mixture by Francis, Piper and Malkin [1930].

Treatment of the unsaponifiable material with phthalic anhydride. The material (45 g.) was heated with phthalic anhydride (45 g.) and pyridine (20 cc.) at 120° for 18 hours and, after removal of pyridine and excess phthalic anhydride in the usual way, the product was dissolved in ether and shaken with a warm solution of sodium carbonate. A copious precipitate of the sodium salts of the primary phthalates was obtained. This was washed in the usual way by shaking with ether and centrifuging, dried in the air and then extracted many times by shaking with ether. The original ethereal extract and the washings were collected, the solvent was removed and the product extracted with boiling alcohol. A small residue (1·7 g.) remained undissolved. The alcoholic solution on cooling deposited a pale green amorphous solid (7·9 g.). The alcohol mother-liquor—which should contain the soluble sodium salts of any secondary alcohol esters—was taken to dryness, but the semi-solid residue (1·2 g.) did not yield any alcohol on saponification. It was presumably a residual amount of that fraction of the unsaponifiable material, the main bulk of which, in the preparation of the crude wax, was soluble in the ether-acetone mixture. The amorphous green powder mentioned above was extracted at room temperature with cold petroleum (B.P. < 40°). The extracts were concentrated and treated with two volumes of acetone; the material which separated (3·15 g.) was removed by filtration, M.P. 59–59·5°. (Found: C, 81·6; H, 13·5 %. Iodine value, 19.) This material, which may contain unsaturated hydrocarbons, is still under investigation.

Primary alcohols. The insoluble white sodium salts of the primary phthalates (45·8 g.) were dissolved in boiling benzene (500 cc.). Sodium ethoxide (25 g. sodium and 350 cc. alcohol) was added and the boiling continued for 5 minutes. The mixture was allowed to cool and to stand for 2 hours. Warm water (500 cc.) was then added and the benzene solution separated in a funnel. The aqueous extract was extracted repeatedly with benzene. The benzene extracts

were combined, the solvent removed by distillation and the brown residue extracted with acetone and ether. There remained an insoluble residue (2·2 g.). The acetone and ethereal extracts gave 27·5 g. of brown crystalline powder (80 % of theory for $C_{26}H_{53}O.CO.C_6H_4CO_2Na$). The crude primary alcohol was redissolved in boiling acetone, clarified with charcoal, and the product which separated on cooling recrystallised first from carbon disulphide and then from acetone; yield 20·7 g. of fine white needles, M.P. 79·3–79·6°. (Found: C, 81·5; H, 14·3. $C_{26}H_{54}O$ requires C, 81·6; H, 14·2 %.) The M.P. of this primary alcohol suggested that it was the usual "ceryl" alcohol which often occurs as a constituent of plant waxes. A sample was oxidised by means of chromium trioxide in acetic acid to the corresponding acid. This had M.P. 86·5–86·8°, some degrees higher than that usually found for the cerotic acid obtained on oxidation of ceryl alcohol. Another sample was reduced *via* the iodide to the corresponding paraffin which melted at 56·3–56·6°. Synthetic *n*-hexacosane has M.P. 56·4–56·6° and, in the early stages of the research, this seemed to us evidence that we were dealing with a fairly pure sample of a primary alcohol containing 26 carbon atoms. At a later period, when our knowledge of the synthetic and mixed paraffins was more complete, it became evident that the paraffin derived from this alcohol may not be pure *n*-hexacosane but a mixture. 2·9 g. of this paraffin were therefore fractionated by means of cold petroleum (B.P. 40–60°) in the way previously described for the resolution of the constituent paraffins of apple wax. Five fractions were finally obtained whose M.P. and crystal spacings are given in Table I. It will be seen that the original paraffin was indeed a mixture but that it consisted in very large part of *n*-hexacosane because,

Table I. *Fractionation of the paraffin obtained from the crude primary alcohol.*

Series number	M.P.	Crystal spacing Å.
205	56·2–56·4°	35·5
206	56·4–56·6°	34·8 32·75
207	56·4–56·6°	35·25 32·2
208	56·4–56·6°	35·1
209	56·5–56·8°	35·5

in two of the fractions, Nos. 206 and 207, the lower spacing for this paraffin appears and the extreme limits of melting-point given by Nos. 205 and 209 do not suggest that much paraffin of higher or lower carbon content was present. Leaving for later discussion the question of whether ceryl alcohol is $C_{26}H_{54}O$ or $C_{27}H_{56}O$ the data given above showed that we were dealing with a far purer sample of *n*-hexacosanol than had hitherto been isolated from natural products. An attempt was therefore made to fractionate this grass alcohol and obtain a specimen of *n*-hexacosanol comparable in melting-point and crystal spacing with the synthetic product.

Fractionation of the primary alcohol. The alcohol (18 g.) was converted into the acetate by boiling overnight with acetic anhydride (250 cc.) and fused

sodium acetate (20 g.). The product was then poured into hot water, stirred mechanically for an hour, collected, taken up in methyl alcohol, boiled with charcoal, and the product finally crystallised from benzene; yield 17·8 g., M.P. 59·7–60°. This acetate was distilled in an apparatus designed specially for the distillation in a high vacuum of substances of high setting-point, which will be described in a separate communication. A Willstätter flask of 100 cc. capacity was employed in the first distillation. At the stage when 6·9 g. remained in the flask the distillation was stopped and the flask replaced by one of 50 cc. capacity. This residual 6·9 g. was then distilled. Throughout both distillations the temperature of the metal-bath was some 70° above that of the vapour passing over. The fractionations obtained are set out fully in Table II in which data concerning the resultant alcohols, and the paraffins, acids and esters derived from them, are also given.

Table II. *Fractionation of the crude primary alcohol.*

No.	B.P./mm.	Wt. g.	M.P.	Alcohol M.P.	Acid M.P.	Ester M.P.	Paraffin M.P.
1	186–189°/0·02	0·51	59·2–59·4°	79·4–79·8°	87·2–87·4°	—	56·2–56·4°
2	190–192°/0·02	1·02	59·4–59·6°	79·5–80°	87·2–87·5°	—	—
3	191·5–192·5°/0·02	2·44	59·4–59·5°	—	—	—	—
4	192–193°/0·017	2·05	59·5–59·6°	79·5–79·8°	87·7–87·9°	59·7–59·8°	56·4–56·6°
5	192°/0·017	1·02	59·4–59·6°	—	—	—	—
6	192–190°/0·017	3·65	59·4–59·6°	—	—	—	—
7	Residue redistilled	6·9	59·8–60°	—	—	—	—
8	196°/0·01	0·66	59·4–59·6°	79·5–79·7°	—	—	—
9	196–193°/0·005	1·04	59·5–59·7°	—	—	—	—
10	193–194°/0·006	1·55	59·3–59·5°	—	—	—	—
11	194–194·5°/0·006	1·5	59·5–59·7°	79–79·5°	86·6–87°	—	—
12	195°/0·005	0·5	59·3–59·5°	—	—	—	—
13	Residue	1·3	59·4–59·7°	78·6–78·8°	84·7–85·3°	—	56·7–56·9°

Each fraction was hydrolysed to the resulting alcohol by saponification with 10 % alcoholic potash for 2 hours, and the white crystalline solid which separated on cooling crystallised from petroleum (B.P. 40–60°). In the case of fractions 3 to 11 inclusive the alcohol, when crystallised from this solvent, came out in large thin leaflets up to 1 cm. in length. Certain of these alcohols (fraction No. 4 will be taken as an example) were then converted into the acids, esters and paraffins in the following way.

The alcohol (0·9 g.) was dissolved in warm glacial acetic acid (40 cc.) and the solution of chromium trioxide (1 g.) in glacial acetic acid (10 cc.) added in small amounts during the course of a quarter of an hour. After standing for a further half-hour the solution was poured into water, which was then extracted with warm benzene. The solvent was removed, the residue taken up in ether and filtered. The ethereal solution was shaken with a solution of sodium ethoxide and the granular sodium salt of the fatty acid separated by centrifuging. This was washed with ether, decomposed with HCl, and the free acid extracted with ether. The resulting product was crystallised four times from acetone, M.P. 86·3–86·8°. (Found: C, 78·4; H, 13·3. $C_{26}H_{52}O_2$ requires C, 78·7; H, 13·2 %.) Recrystallised from pyridine and then four times successively from acetone,

the acid melted at 87·7–87·9°. The ethyl ester, recrystallised repeatedly from acetone, was obtained in long flat needles about 1 cm. in length, M.P. 59·7–59·8°. (Found: C, 79·2; H, 13·5. $C_{28}H_{56}O_2$ requires C, 79·3; H, 13·3 %.)

Another portion of the alcohol was converted into the iodide. This was reduced to the corresponding paraffin by a method used by Levene *et al.* [1915] in place of that previously employed by us, *viz.* amalgamated zinc wool and alcoholic HCl. The iodide (0·9 g.) was dissolved in glacial acetic acid (10 cc.), zinc dust (1 g.) added, and the mixture boiled over a small free flame. Every two hours a stream of dry HCl was passed in for about half a minute. At the end of two working days the zinc had dissolved. The mixture was diluted with water and a layer of paraffin (0·6 g.) collected. This was purified by treatment with sulphuric acid at 130° and crystallised from benzene-alcohol (1 : 2), M.P. 56·4–56·6°. This paraffin was then fractionated by means of cold petroleum (B.P. 40–60°) into six fractions whose M.P. and crystal spacings are given in Table III. The most striking feature of the results is the appearance of the

Table III. *Fractionation of the paraffin obtained from the purified primary alcohol.*

Series number	Transition-point on melting	M.P.	S.P.	Transition-point on cooling	Crystal spacing A.		
					A	*B*	*C*
B 72	50·5–51·5°	56·4–56·6°	56·2°	48·1°	34·6	—	30·9
B 73	51–52°	56·4–56·6°	56·1°	48·2°	—	32·65	30·9
B 74	51–52°	56·4–56·6°	56·1°	48·2°	—	—	30·9
B 75	51–52°	56·4–56·6°	56·1°	48·2°	—	—	30·9
B 76	51–52°	56·4–56·6°	56·1°	48·2°	—	—	31·0
B 77	51–52°	56·4–56·6°	56·1°	48·1°	—	32·6	31·0
$C_{26}H_{54}$	51·5–52°	56·4–56·6°	56·1°	48·3°	34·95	32·6*	31·0

spacing in the column headed *B*. Although such a spacing has not been observed with synthetic hexacosane the value 32·6 falls exactly at the right place on the extrapolated *B* line. The extrapolated value for the *A* (vertical chain spacing) of $C_{24}H_{50}$ is 32·5. Now $C_{26}H_{54}$ is the first of the long paraffins to fail to appear in the *B* modification. It is therefore likely that the presence of a small impurity, having a normal spacing with the value which hexacosane would take in the *B* modification, will be able to force this crystal form upon it. We conclude that there is a small contamination of $C_{24}H_{50}$. The depression of the transition-point on cooling for five of the middle fractions is only 0·1°. As a comparison we know that 1 % of hexacosane in octacosane depresses the transition-point on cooling by 0·6° and on heating by 0·5°. We are therefore confident that there is much less than 1 % of tetracosane in these mixtures.

If this is so then the alcohol of Fraction 4 is *n*-hexacosanol with less than 1 % *n*-tetracosanol. Unfortunately at the present time this alcohol has not been synthesised, so that direct confirmatory data are lacking. We can, however, obtain further evidence for the purity of the grass product by considering the M.P. and crystal spacings of the alcohol itself and the derived acid and its ester. The alcohol melted at 79·8° and the crystal spacing (Series B, No. 33) was

* "Extrapolated value."

57·8 Å. From the data of Malkin [1930] extrapolated values for *n*-hexacosanol would be M.P. about 80° and crystal spacing 57·9 Å. The acid melted at 87·8°. The M.P. of synthetic *n*-hexacosanic acid was found by Francis, Piper and Malkin [1930] to be 88·2°, but they state quite clearly that this point is 0·5° higher than they would expect from their curve showing the M.P. of the even number acids. The crystal spacings for the synthetic acid were 63·3 Å. for the *B* and 57·3 Å. for the *C* spacings. The spacings obtained for the acid derived from the grass alcohol were (Series B, No. 35) 62·5 Å. and 56·7 Å. respectively. The synthetic ethyl ester melted at 59·6° and had a crystal spacing of 34·64 Å. The ethyl ester derived from the grass alcohol melted at 59·8° and had a crystal spacing (Series B, No. 37) of 34·4 Å.

There are no data for mixed even number acids of more than 18 carbon atoms, but it is to be expected that a shorter chain would lower both *B* and *C* spacings of hexacosanic acid. The results of the measurements on the acid from the grass alcohol are to this extent in accord with the deductions made for the derived paraffin—namely, that a small amount of tetracosanic acid is present. The hexacosanic acid was not available in sufficient amount to purify *via* the ester, and cannot therefore be fairly compared with that of Francis, which had been through this process. Similar conclusions may be drawn from the spacings of the ester.

The alcohol of Fraction 1, M.P. 79·4–79·6°, gave an acid, M.P. 87·2–87·4°, and a paraffin, M.P. 56·2–56·3°, which was fractionated with light petroleum. The results are given in Table IV. The high M.P. of the acid and the narrow

Table IV. *Fractionation of the paraffin obtained from the highest and lowest boiling fractions of the crude primary alcohol acetates* (cf. *Table II*).

Fraction number	Series number	Transition-point	M.P.	S.P.	Crystal spacings Å. *A*	*B*	*C*
1	B 101	51–52°	56·3–56·5°	56·0°	—	32·6	—
	B 102	49–50°	56·2–56·4°	55·8°	—	32·6	—
	B 103	48·5–51·5°	56·2–56·3°	55·8°	30·8	—	34·6
	B 104	48–50°	56·2–56·4°	55·8°	30·8	—	34·6
13	B 105	43–44°	56·8–57°	56·8°	—	—	—
	B 106	43–45°	56·8–57·1°	56·6°	—	—	—
	B 107	43–45°	56·7–57°	56·5°	—	—	—
	B 108	44·5–45·5°	56·4–56·7°	56·4°	—	—	—

range within which the paraffins melt show that this lower fraction consists in very large part of *n*-hexacosanol. The *B* spacing again suggests the presence of a small amount of $C_{24}H_{50}$, about 1 % in B 103 and 104, much less in B 101 and 102. It is interesting to note that the depression in the transition-temperature of about 2° with a change in M.P. of only 0·1–0·2° would have suggested to us, from a consideration of the data given in the preceding paper, that the impurity was not *n*-pentacosane but tetracosane (compare series for C_{28}–C_{29} and C_{26}–C_{28}). The alcohol from the residue (Fraction 13) obviously contained alcohols higher than *n*-hexacosanol. The low transition-temperatures recorded

in Table IV for the derived paraffins show that these contain a small amount of a mixture of at least two other higher paraffins, in the case of B 105 as much as 10 %.

Perennial ryegrass (Lolium perenne).

Preparation and saponification of the wax. The wax was prepared and treated in a similar way to that described for the products from cocksfoot. In the case of the ryegrass the wax amounted to only 10 % of the total ethereal extract, or 0·38 % of the dry weight of the grass.

14·3 g. of the wax were saponified as before and yielded 2·6 g. of fatty acids, M.P. 80–81°, together with an unsaponifiable fraction which, after crystallisation from acetone and decolorising with charcoal, weighed 8·6 g. This material yielded an insoluble sodium salt of a primary phthalate (6·8 g.) and a crude paraffin fraction (2·1 g.).

Primary alcohol. The insoluble sodium salt was hydrolysed with sodium ethoxide in benzene-alcohol and yielded 4·2 g. of white crystalline primary alcohol, M.P. 78°. Fractional crystallisation from light petroleum, carbon disulphide and acetone gave two products.

(1) (2·4 g.) M.P. 79·8–80°; crystal spacing (Series C, No. 57), 58·4 Å. It gave on oxidation an acid, M.P. 85–85·5°, and on reduction *via* the iodide a paraffin, M.P. 56·5–56·7°; crystal spacing (Series C, No. 58), 35·5 Å.

(2) (1·2 g.) M.P. 79–79·3°; crystal spacing (Series C, No. 39), 58·2 Å.; acetate, M.P. 60·2–60·5°; acid, M.P. 84·5–84·8°; and paraffin, M.P. 56·4–56·6°. The crystal spacings of this paraffin (Series C, No. 24) were 35·5 and 32·5 Å.

The M.P. and crystal spacings of the derived paraffins suggest that these alcohol fractions consist in large part of *n*-hexacosanol, but the amount of longer-chain alcohols present is greater than in the case of the corresponding product from cocksfoot.

Paraffin fraction. No trace of a secondary alcohol or a ketone could be found. The crude paraffin was decolorised with charcoal in 95 % alcohol and then treated with sulphuric acid; M.P. 65·9–66°, transition-temperature indistinct. Fractional crystallisation from petroleum gave without any trouble the samples recorded in Table V. It will be seen that the paraffin is a complex

Table V. *Fractionation of the paraffin, M.P. 66°, isolated from ryegrass.*

Series number	Heating transition-point	M.P.	S.P.	Cooling transition-point	Crystal spacing Å.
B 22	50·5–51·5°	62·3–62·6°	62·0°	50–49·5°	39·7
B 23	55–56°	65·0–65·5°	64·5°	54–53°	40·75
B 24	57–58°	66·7–66·8°	66·5°	57–56°	42·3
B 25	60–61°	67·9–68·1°	67·8°	60–59°	43·0
B 26	61–62°	68·4–68·8°	68·4°	61–60°	43·5

mixture and its composition cannot even be suggested. The amount isolated was small, and as it is impossible to obtain large samples of a given species of grass which can be considered free from all other grasses we do not consider

the present wax a suitable raw material for experiments on the separation of mixed paraffins in plant waxes. The fact that the properties of this crude paraffin agree with those ascribed by many writers to *n*-triacontane has been briefly mentioned in the Introduction.

Discussion.

It will be seen that the fractionation of these grass waxes has yielded in large part a fairly pure specimen of a long-chain primary alcohol. There were also obtained certain products whose nature has not yet been definitely determined and only a small amount of higher fatty acids. The amount of true wax, therefore, must have been relatively small, and the major part of the primary alcohol must have been uncombined. The fact that this primary alcohol was practically a pure specimen of *n*-hexacosanol allows us to discuss in some detail the constitution of the alcohol termed "ceryl," which was originally isolated from Chinese wax, and the hippocoprosterol of Bondzynski and von Humnicki [1896].

Ceryl alcohol. The data recorded by previous workers are set out fairly fully in Table VI. There are numerous other references to the isolation of this

Table VI. *Collected data concerning ceryl alcohol.*

Reference	Source	Alcohol M.P.	Acetate M.P.	Acid M.P.	Ester M.P.	Paraffin M.P.
This paper	Cocksfoot crude alcohol	79·6°	59·7–60°	86·8°	—	56·3–56·6°
This paper	Cocksfoot purified alcohol*	79·7°	59·5–59·6°	87·7–87·9°	59·7–59·9°	56·4–56·6°
This paper	Perennial ryegrass	79·3°	60·2°	84·5–84·8°	—	56·5–56·7°
Francis *et al.* [1930]	Chinese wax	79°	60°	82°	60°	—
"	"	83°	65–66°	83·8°	64·2°	—
Levene *et al.* [1915]	"	80°	64–65°	—	—	61°
Gascard [1921]	"	79·5–80°	—	82–82·5°	—	59·5°
This paper	"	78·7°	62°	83–83·2°	—	59–59·5°
Brodie [1848]	Beeswax	79°	—	81°	—	—
Schwalb [1886]	"	79°	—	78·5°	—	—
Stürcke [1884]	Carnauba wax	76°	—	—	—	—
Benedikt and Ulzer [1866]	Seed lac	79·5°	65°	—	—	—
Collison and MacLean [1931]	Spinach leaves	77–78°	61·5°	—	—	—
Stern and Zellner [1926]	Bark of *Sonchus arvensis*	80°	63·4°	—	—	—
Bareuther [1923]	Sunflower oil	81·2°	63·5°	77·6°	—	—
Chibnall *et al.* [1931]	Apple-peel wax	80·2–80·7°	64·5–64·8°	83–83·5°	—	60–60·2°

* Shown to be *n*-hexacosanol with less than 1 % impurity.

alcohol from plant sources, but in these cases the M.P. of derivatives have not been quoted. The M.P. of the alcohol was generally recorded as 79–80°, of the acetate as 64–65°, and of the acid obtained on oxidation as 81–82°. Titration of the acid suggested to earlier workers that it contained 26 carbon atoms. The low M.P., however, was a puzzle because the extrapolated melting-point of *n*-$C_{26}H_{52}O_2$ was many degrees higher than that actually found. Many workers, therefore, suggested that this and similar alcohols, such as myricyl alcohol, contained a branched chain, and that this was the reason for the low M.P. of the acid. Levene *et al.* [1915] obtained a specimen of ceryl alcohol, M.P. 80°, giving an acetate, M.P. 64–65°, and by reduction of the corresponding iodide a paraffin,

M.P. 61°. This latter was higher than that given by synthetic *n*-hexacosane and they concluded that the ceryl alcohol must have a branched and not a normal carbon chain. A more complete investigation of ceryl alcohol from Chinese wax was made by Gascard [1921]. By fractionation he obtained what he thought was pure ceryl alcohol, M.P. 79·5–80°, which yielded on oxidation a specimen of cerotic acid, M.P. 82–82·5°. The molecular weight of this acid by titration suggested that it contained 27 carbon atoms. To confirm this finding he reduced the alcohol *via* the iodide to the corresponding paraffin. This melted at 59·5°, the same temperature as the M.P. of *n*-heptacosane previously synthesised by Kraft. Gascard, therefore, concluded that ceryl alcohol was $C_{27}H_{56}O$.

More recently, Francis, Piper and Malkin [1930] fractionated, by distillation of the acetate, a specimen of ceryl alcohol from Chinese wax and separated two samples, one melting at 79° and the other at 83°. The corresponding acids melted at 82° and 83·8° respectively. An examination of the crystal spacings of the acids suggested that the former was a mixture of C_{26} and C_{28}, while the latter was C_{28} with probably an even higher acid. No evidence of branched chain acids was obtained, and they concluded that ceryl alcohol from Chinese wax was a mixture of primary alcohols. With this finding we are in complete agreement, and our own researches enabled us to reconcile some of the earlier conflicting results.

In the first place a specimen of ceryl alcohol, M.P. 79·7°, obtained from Chinese wax, gave an acetate, M.P. 62°, an acid, M.P. 83–83·2°, and a paraffin, M.P. 59–59·5°. These values it will be observed are in agreement with those of Gascard. The crystal spacing (Series C, No. 44) of the paraffin, however, gave only three very diffuse orders measuring 36·7 Å. It was clearly a mixture of more than two paraffins, showing that the ceryl alcohol was itself a complex mixture of mean molecular weight corresponding to C_{27}.

We have in the present research isolated what the earlier workers obviously considered to be ceryl alcohol. Table V shows that the M.P. of the acid derived from both the crude and purified grass alcohol is some 4° higher than that recorded by any previous worker. The acetate, on the contrary, melts some 2–4° lower. As the grass alcohol has been shown to be nearly pure *n*-hexacosanol it is clear that all the other alcohols quoted in Table V were mixtures of alcohols which, on oxidation, have given a mixture of acids showing a depressed M.P. Data as to the melting-points of the pure and mixed acetates of the higher primary alcohols are lacking, but it is clear that those of mixed acetates exhibit very much less depression than those of the corresponding acids.

The actual isolation of *n*-hexacosanol from natural sources and the definite proof that the ceryl alcohol of former workers was neither *n*-hexacosanol nor *n*-heptacosanol but a mixture of primary alcohols of the *n*-fatty acids necessitate a new definition of the name ceryl alcohol. Applied originally to the alcohol, M.P. 80°, isolated from Chinese wax, it has unfortunately always been applied to products now shown to be mixtures. Such mixtures will no doubt be encountered in future researches and a collective name will still be required

for them. We suggest that the name ceryl alcohol be retained for this purpose and that, if further fractionation should lead to the isolation of pure primary alcohols of the *n*-fatty acids, these should be referred to by their chemical names. On this new definition the alcohol isolated in the present research from cocksfoot is *n*-hexacosanol and not ceryl alcohol.

Hippocoprosterol. This substance, M.P. 74·5°, was discovered by Bondzynski and von Humnicki [1896] in the faeces of the horse and was supposed to have been formed by reduction of cholesterol in the intestine. The product was further examined by Wilenko [1906] who, by fractional crystallisation from 97 % alcohol, obtained two isomeric compounds, M.P. 67° and 56°, which he designated as α- and β-hippocholesterol respectively. Dorée and Gardner [1908] were unable to substantiate Wilenko's findings and showed that the hippocoprosterol was a saturated alcohol, M.P. 79°, which gave an acetate, M.P. 61–61·2°, and no sterol reactions. The same material was found in the excrement of the cow and sheep and also in the ether extract of grass itself. They therefore drew the conclusion that hippocoprosterol was not a product of animal metabolism but was a constituent of the grass taken as food which had passed unchanged through the animal.

From the results given in the present paper there is no doubt that hippocoprosterol, M.P. 79°, acetate, M.P. 61·2°, is ceryl alcohol. Ordinary forage grass is a mixed herbage, and there is no reason to suppose that all the component grasses contain nearly pure *n*-hexacosanol as do cocksfoot and perennial ryegrass. Furthermore some of these grasses may contain a larger quantity of a paraffin than ryegrass. A mixture of paraffins and ceryl alcohol is not readily separated by crystallisation from alcohol, and in that fact seems to us to lie the explanation of Wilenko's low M.P. We attribute the differences between Dorée and Gardner's and Wilenko's results therefore to a difference in the herbage mixture.

Summary.

The wax fractions of two common forage grasses, cocksfoot and perennial ryegrass, have been investigated.

In the case of cocksfoot the chief constituent is a long-chain primary alcohol which has been shown to be nearly pure *n*-hexacosanol. It contains less than 1 % of *n*-tetracosanol, and about an equal amount of an unidentified longer-chain alcohol.

The same alcohol is present in ryegrass wax, but the amount of impurities is greater.

The constitution of the so-called ceryl alcohol is discussed, and the recommendation made that the term be restricted in future to the mixed primary alcohols, M.P. *ca.* 80°, which often occur in plant waxes.

The probable identity of hippocoprosterol with ceryl alcohol is discussed.

From previous work on hippocoprosterol it is concluded that the wax fractions of both these forage grasses have no feeding value.

We should like to record our thanks to the Imperial Chemical Industries for a grant to cover the cost of this research, and their staff at Jealott's Hill Research Station, Bracknell, for the supply of pure strain grasses.

REFERENCES.

Bareuther (1923). *Chem. Umschau.* **30**, 117.
Benedikt and Ulzer (1866). *Monatsh.* **9**, 581.
Bondzynski and von Humnicki (1896). *Z. physiol. Chem.* **22**, 396.
Brodie (1848). *Liebig's Ann.* **67**, 180.
Chibnall and Channon (1927). *Biochem. J.* **21**, 225.
—— Piper, Pollard, Smith and Williams (1931). *Biochem. J.* **25**, 2095.
Collison and MacLean (1931). *Biochem. J.* **25**, 2, 606.
Dorée and Gardner (1908). *Proc. Roy. Soc. Lond.* B **80**, 212.
Francis, Piper and Malkin (1930). *Proc. Roy. Soc. Lond.* A **128**, 214.
Gascard (1921). *Ann. Chim.* (9), **15**, 332.
Levene, West and Van der Scheer (1915). *J. Biol. Chem.* **20**, 521.
Malkin (1930). *J. Amer. Chem. Soc.* **52**, 3739.
Schwalb (1886). *Liebig's Ann.* **235**, 106.
Stern and Zellner (1926). *Monatsh.* **46**, 461.
Stürcke (1884). *Liebig's Ann.* **223**, 283.
Wilenko (1906). *Bull. Internat. Acad. Sci. Cracovie*, **1**, 20.

CCXXVIII. THE DICARBOXYLIC ACID-NITROGEN OF PROTEINS.

WITH A NOTE ON THE ALCOHOL-SOLUBLE PROTEIN FROM RAGI (*ELEUSINE CORACANA*).

By MANAYATH DAMODARAN.

From the Biochemical Department, Imperial College of Science and Technology, South Kensington.

(*Received October 31st, 1931.*)

The usual procedure for the Van Slyke determination of the nitrogen distribution of proteins [Plimmer, 1917] makes no provision for the dicarboxylic acids. A modification was described by Andersen and Roed-Muller [1915] in which the dicarboxylic acids were determined indirectly by the base bound at neutrality to phenolphthalein. With the only two proteins investigated by these authors the method gave satisfactory results but, involving as it does the complete absence of calcium salts and the previous removal of the diamino-acids, it calls for considerable modification of the ordinary Van Slyke procedure by which all the known proteins have been analysed. The presence of ammonia, of glucosamine or of organic acids was also shown to give rise to error. Probably for these reasons the method of Andersen and Roed-Muller has found no general application.

In the course of many protein analyses carried out in this laboratory it has been found that the lime-alcohol method of Foreman [1914] for precipitating the dicarboxylic acids gave very satisfactory results and that it could be easily incorporated into the ordinary procedure for Van Slyke analyses, so that the total dicarboxylic acids could be rapidly determined when working with only small quantities of material and without actual isolation of the amino-acids themselves.

The addition of this new group to the usual nitrogen distribution scheme is considered all the more desirable in view of the many errors to which the determination of the bases has been shown to be liable. The separation of the bases by means of phosphotungstic acid is neither quantitative nor specific [Thimann, 1930; Plimmer and Rosedale, 1925, 1; Gortner and Sandstrøm, 1925]. According to Plimmer [1916], the decomposition of histidine, amounting to 3–4 %, tends to give a higher value for arginine. To this must be added the possible presence in protein hydrolysates of the newly discovered amino-acid, citrulline [Wada, 1930], which also decomposes, giving ammonia on treatment with alkali. Finally it has to be remembered that peptides and, under certain

conditions, amino-acids such as phenylalanine and proline, are precipitated by phosphotungstic acid.

Apart from widening the scope of the Van Slyke method of analysis and providing an additional criterion for the characterisation of proteins in general, a knowledge of the dicarboxylic acid content of plant proteins is of importance on account of the possibly predominant rôle played by asparagine and glutamine in plant metabolism [Onslow, 1930].

The technique previously adopted in this laboratory was the modification of the method of Foreman [1914] described by Kingston and Schryver [1924] wherein barium hydroxide was substituted for calcium hydroxide. This modification is convenient in dealing with large amounts of protein as the quantity of alcohol required is much smaller, but it has the disadvantage that the amount of extraneous matter precipitated is appreciable [Jones and Moeller, 1928; Damodaran, 1931]. It was therefore decided to attempt to apply Foreman's original procedure to the Van Slyke method of small scale analyses.

Experiments were carried out on the following lines. (i) The completeness of the precipitation by alcohol of the calcium salts of glutamic and aspartic acids when these were present in small amount was tested. (ii) As the dicarboxylic acid precipitate is known to be contaminated with a small amount of material precipitable by phosphotungstic acid it was necessary to see if due allowance could easily be made for such material and a reasonably correct figure thus obtained for the basic N. (iii) When protein hydrolysates are treated by the Foreman method it is found that, after apparently complete precipitation of the dicarboxylic acids (as tested by adding small amounts of alcohol), a further flocculent precipitate always forms slowly either on standing for a long time or on the addition of large volumes of alcohol. With caseinogen Foreman showed that this precipitate contained no dicarboxylic acids. It was considered desirable to verify this with at least one other protein of a different class and to this end experiments were carried out on gliadin. (iv) Experiments were made on gliadin to see if reprecipitation were necessary when using calcium hydroxide. (v) The new modification was finally tested on five proteins whose dicarboxylic acid contents have been definitely determined by large scale analyses and was then applied to a number of proteins of different classes, the dicarboxylic acid contents of which are either unknown or not accurately known.

Experimental.

Completeness of the precipitation of glutamic and aspartic acids by calcium hydroxide and alcohol. 5 cc. of a solution of glutamic acid hydrochloride containing 0·1 g. of glutamic acid were made alkaline with calcium hydroxide and treated with 95 % alcohol till precipitation was complete, approximately 50 cc. being used. The precipitate was filtered at the pump and washed three times with 20 cc. portions of 95 % alcohol; 98·8 % of the glutamic acid was precipitated. A similar experiment with 5 cc. of a 0·2 % solution of aspartic acid showed that 99·1 % of the acid was precipitated.

Analysis of gliadin. It will be convenient here to describe in full the modified Foreman method as applied to gliadin.

Hydrolysis. 6 g. of gliadin (for duplicate analyses) were hydrolysed by boiling with 20 % hydrochloric acid for 18 hours. On an aliquot total N and amide-N were determined.

Amide-N. It was found convenient to determine the amide-N by distilling in steam a small aliquot of the hydrolysate with sodium hydroxide in a Parnas-Wagner micro-distillation apparatus for 4½ minutes. In most cases slightly higher results were obtained than those previously recorded, but this can hardly be ascribed to decomposition of arginine or other amino-acids as the period of distillation involved is so short.

The main fraction of the hydrolysate was next divided into two portions each of which was treated in the following way.

Removal of humin and ammonia. The solution was made strongly alkaline with calcium hydroxide (10 % suspension), the precipitated humin with the excess of calcium oxide centrifuged off and washed free from chlorides (three washings each with 50 cc. of warm water were usually required). The solution with the washings was evaporated *in vacuo* at 40° to a volume of 10–15 cc. The air admitted during distillation was freed from carbon dioxide by means of a soda-lime tube.

Precipitation of the dicarboxylic acids. To the syrup in the flask about 10 vols. of 95 % alcohol were added very slowly in small quantities at a time, with vigorous stirring, till precipitation was complete. This point was determined by transferring the mixture to a 250 cc. centrifuge-tube, centrifuging and testing by the addition of 10 cc. portions of alcohol. When no further precipitate formed the clear liquid was poured off and the precipitate, some of which adhered to the sides of the flask, was washed twice with 50 cc. portions of 95 % alcohol.

Treatment of the dicarboxylic precipitate. The precipitate was dissolved in a little acidulated water and the solution after making up to 25 cc. was analysed for nitrogen. The value obtained was 28·6 % of the total N. The remainder of the solution was transferred to a centrifuge-tube, and treated with 5 cc. of concentrated hydrochloric acid and a saturated aqueous solution of 5 g. of phosphotungstic acid. The precipitate formed was redissolved by warming in a water-bath and allowed to crystallise at room temperature for 2 days. It was then separated by centrifuging, washed with a solution of 2·5 g. phosphotungstic acid and 3·5 g. hydrochloric acid in 100 cc. water, and finally dissolved in a strong solution of sodium hydroxide. Nitrogen was determined on an aliquot. The basic material thus separated from the dicarboxylic acid precipitate contained 0·6 % of the total N. Allowing for this the value for the dicarboxylic acid-N was 28·0 %.

Precipitation of the bases. The alcoholic mother-liquor and washings from the calcium dicarboxylates were made neutral with acetic acid, evaporated *in vacuo* to remove all alcohol and made up to a volume of 50 cc. The precipitation

with phosphotungstic acid was now carried out according to the Van Slyke procedure, except that the precipitate was separated by centrifuging instead of by filtration. It was dissolved in a strong solution of sodium hydroxide and nitrogen was determined on an aliquot.

The mother-liquor and washings were treated as in the usual Van Slyke analysis and both total N and amino-N determined.

Effect of the precipitation of the dicarboxylic acids on the "basic" N. Parallel with the analysis of gliadin already described another determination was made in which the normal Van Slyke procedure was followed, the bases being precipitated directly after the removal of ammonia. The value thus obtained was 10·7 %. No solubility correction was applied in view of the recent work of Thimann [1930] on the bases. In the first analysis, the basic material precipitated from the dicarboxylic acid fraction was 0·6 % (in terms of total N) and 10·2 % from the remaining solution, thus giving a total value of 10·8 %. The agreement is considered sufficiently good for present purposes.

Similar experiments were made with certain other proteins, the basic N being determined in one case directly and in the other after precipitation of the dicarboxylic acids. It was found that varying amounts of basic material were removed with the dicarboxylic acids, a maximum of 2·2 % of the total N being found with caseinogen. Experience suggests that the governing factor is not the percentage of the bases present in the protein, but the physical condition of the precipitate and that by adding the alcohol in small quantities with vigorous shaking the amount of basic impurities could be reduced to a minimum. In all cases the bases were quantitatively recovered by precipitating the dicarboxylic acid solution itself with phosphotungstic acid.

Examination of the small precipitate obtained on adding large volumes of alcohol to the solution from which the calcium dicarboxylates had been precipitated as above. 10 g. of gliadin were hydrolysed and dicarboxylic acids separated as before. To the mother-liquid thus obtained an equal volume of alcohol was added and the solution kept overnight. The precipitate was filtered off and dissolved in the minimal amount of concentrated hydrochloric acid; the solution was saturated with hydrogen chloride gas and kept in the ice-chest. No separation of glutamic acid hydrochloride was observed. A similar small precipitate obtained from another 10 g. of gliadin hydrolysate was treated with copper oxide. No insoluble copper salt was obtained. These results confirm those of Foreman with caseinogen.

Effect of dissolving the dicarboxylic acid fraction and reprecipitating with alcohol. The precipitate obtained from gliadin with calcium hydroxide and alcohol, as already described, which contained 28·0 % of the total nitrogen was dissolved in about 15 cc. of water and reprecipitated by the addition of alcohol as before. The nitrogen content of the second precipitate thus obtained was 27·7 % of the total protein-N, showing that the amount of impurities removed by a second precipitation was inconsiderable.

Analysis of various proteins.

Of the proteins analysed the following were prepared in the laboratory according to the usual methods: gliadin, zein, edestin (from hemp-seed), haemoglobin and serum-globulin (from horse-blood). B.D.H. preparations of the following proteins were used: caseinogen, egg-albumin, pepsin. The leaf proteins analysed were the original preparations of these proteins made by Prof. Chibnall to whom I am indebted for their gift.

The nitrogen contents of the preparations used (calculated on an ash- and moisture-free basis) are given in column 3 of Table I. Though complete analyses were carried out on most of the proteins examined only the figures relevant to the present purpose are quoted. The amide-N figures are included in every case on account of their significance with regard to the relationship that is usually assumed to exist between the ammonia and the dicarboxylic acids resulting from the acid hydrolysis of proteins.

Table I. *Analysis of certain proteins.*

All figures given are in percentages of total protein nitrogen.
(A, present analyses. B, other workers*.)

							Basic N				
							A				
								Indirectly precipitated			
			Amide-N		Dicarboxyl c acid-N		Directly	In dicarb-	In		
	Protein	Total N	A	B	A	B	pre-cipitated	oxylic acid fraction	main solution	Total	B
Proteins recently analysed for their dicarboxylic acid content:											
1	Gliadin	17·3	25·7	25·52 24·61 26·13 25·9	28·0	26·79(1)	10·7	0·61	10·2	10·8	7·26 5·61 12·91 10·97
2	Edestin	18·5	10·2	9·99	15·0	15·6(1)	35·1	1·84	32·63	34·5	38·15 31·7
3	Caseinogen	14·6	10·3	10·31 10·43	15·4	14·8(2)	23·1	2·20	20·45	22·7	24·12
4	Egg-albumin	14·9	8·8	8·65	14·7	12·3(1)	24·2	0·84	23·09	23·9	21·27
5	Zein	16·0	19·6	20·75	17·5	18·56(3)	—	0·56	2·90	3·5	3·1
Other proteins:											
6	Haemoglobin	16·9	5·6	5·24 6·37	9·4	—	—	0·44	28·80	29·2	31·3
7	Serum-globulin	15·9	8·1	—	13·1	—	—	1·21	22·77	24·0	25·25
8	Pepsin	13·4	3·6	—	12·3	—	—	0·35	21·2	21·6	—
Leaf proteins:											
9	Spinacin	16·3	—	6·93(4)	17·7						
10	*Zea Mays*	14·4	—	7·44(4)	21·9						
11	Runner bean	15·9	—	5·69(4)	20·8						
12	Alfalfa	15·7	—	5·5(4)	20·1						

(1) Jones and Moeller [1928]. (2) Foreman [1914]. (3) Dakin [1923]. (4) Chibnall and Grover [1926].

* Except where specific reference numbers are given all data in columns B are taken from Plimmer [1917], or Hoffmann and Gortner [1925].

Discussion.

The values for the dicarboxylic acids for the proteins 1–5 given by the method now described are in close agreement with those of previous workers who have used modern methods for the direct isolation of the acids themselves, and show that a good insight into the dicarboxylic acid content of proteins can be obtained by this simple addition to the usual procedure of Van Slyke. The values for the basic N determined directly and indirectly also show that the modification introduces no appreciable error in the determination of this group.

It is interesting to re-examine in the light of the analytical results now recorded the close agreement found by Osborne, Leavenworth and Brautlecht [1908] between the amide-N of proteins and their content of dicarboxylic acids. Assuming that one carboxyl of the acids was present as an amide group they calculated the proportion of ammonia which should be liberated by acid hydrolysis and compared this with the amount actually found. In Table II their results, recalculated to conform with the data given in Table I, are compared with those now available. Whereas Osborne found the agreement was fair in most cases, in others, especially the prolamins, the divergence was wide. The discovery of hydroxyglutamic acid and improvements in the methods for estimating glutamic and aspartic acids have so increased the total dicarboxylic acid content that the prolamins now constitute the only group which shows anything approaching agreement; the others all have an excess of dicarboxylic acids. This is very marked in the case of the leaf proteins which exhibit, as a class, the same regularity in dicarboxylic acid content as Chibnall and Grover [1926] found for the bases. Such a close agreement in amino-acid composition is not found in any class of seed proteins.

Table II.

Protein	Dicarboxylic N (Osborne)	Dicarboxylic N (from Table I)	Amide-N (Osborne)
Edestin	9·68	15·6	9·99
Glutenin	13·33	16·4[1]	18·87
Caseinogen... ...	7·28	15·4	8·49
Ovalbumin... ...	7·12	14·7	8·66
Gliadin	20·47	28·0	24·61
Zein...	11·69	18·56	18·44
Hordein	20·1	23·6[2]	23·31
Prolamin from Ragi	—	22·3[3]	21·4[3]

[1] Damodaran [1931]. [2] Kleinschmidt [1907]. [3] Damodaran, this paper.

In view of the large margin of error in calculating the values for the different amino-acids in the basic precipitate it is tentatively suggested that the determination of the following groups would give a reliable distribution of nitrogen for the characterisation of proteins:

(i) Amide-N.
(ii) Humin-N.
(iii) Dicarboxylic acid-N.
(iv) Basic N.

(v) Arginine-N, determined directly according to Plimmer and Rosedale [1925, 2].

(vi) Non-amino-N.

(vii) Monoaminomonocarboxylic acid-N.

NOTE ON THE ALCOHOL-SOLUBLE PROTEIN FROM RAGI (*ELEUSINE CORACANA*).

Ragi (*Eleusine coracana*) forms the staple cereal food of the people in some of the drier parts of India. It was hoped to make a study of all the proteins in this seed, but unfortunately only one, a prolamin, could be prepared in a state of purity.

The average protein content of the seed was 6·8 % (N × 6·35) and it consisted of a globulin, a prolamin, and a glutenin. From the finely ground ether-extracted seed a 9 % solution of sodium chloride (found by trial to be the optimal strength) extracted 29·2 % of the total N. The residue after being washed free from sodium chloride and air-dried was exhaustively extracted with warm 73 % alcohol to dissolve out the prolamin. 40·2 % of the total N was removed by this treatment. The residual material was finally treated with 0·4 % sodium hydroxide which dissolved a further 12·6 % of the N. 82·8 % of the seed-N was thus accounted for. Attempts to isolate the globulin by dialysis of the sodium chloride extract and of the glutenin by precipitation from the sodium hydroxide solution gave products which were impure and contained only about 12 % N. Variations in the method, such as treatment with takadiastase to remove carbohydrate, and extraction with alcoholic potash as recommended by Jones and Csonka [1927] were tried without success.

Experimental.

It was found that the quickest and most satisfactory method for preparing the prolamin was to extract the finely ground seed with boiling 73 % alcohol for 1 hour, filter and evaporate *in vacuo* to small volume. By preventing the alcoholic strength from going much below 70 %, either by the addition of further portions of the extract or of alcohol, the extract could be concentrated to a thick syrup without causing any denaturation of the protein. The syrupy solution so obtained was poured with vigorous stirring, while still warm, into about 20 volumes of acetone, when the protein was precipitated as a fine powder.

The substance, after filtering at the pump and drying in air, was purified by redissolving in a small volume of warm 73 % alcohol and reprecipitating with acetone as before. The air-dried powder contained 6·1 % moisture, 0·9 % ash, and moisture- and ash-free, 16·4 % N. A portion was redissolved in alcohol and again precipitated with acetone; but the nitrogen content was not

thereby increased. Tests for sulphur were negative. The isoelectric point of the protein, determined approximately by dialysing a suspension in distilled water in a cellophane bag for 48 hours, and measuring electrometrically the minimal p_H attained, gave a value between p_H 4·8 and p_H 5·0.

The prolamin was analysed by the method outlined in this paper.

Nitrogen distribution.

	% total N
Humin-N	0·8
Amide-N	21·4
Dicarboxylic acid-N...	22·5
Basic N	5·8
Arginine-N	2·2
Monoamino-monocarboxylic acid-N	44·1
Non-amino-N	5·1

It will be seen that the protein has an amino-acid composition characteristic of the prolamins. The amide-N and dicarboxylic acid-N are high, and in close agreement, while the basic N is relatively low [Damodaran, 1931].

My thanks are due to Prof. A. C. Chibnall for the interest he has taken in this work and for much valuable advice.

REFERENCES.

Andersen and Roed-Muller (1915). *Biochem. Z.* **73**, 326.
Chibnall and Grover (1926). *Biochem. J.* **20**, 113.
Dakin (1923). *Z. physiol. Chem.* **130**, 159.
Damodaran (1931). *Biochem. J.* **25**, 190.
Foreman (1914). *Biochem. J.* **8**, 463.
Gortner and Sandstrøm (1925). *J. Amer. Chem. Soc.* **47**, 1663.
Hoffmann and Gortner (1925). Colloid Symposium Monograph, **2**, 209.
Jones and Csonka (1927). *J. Biol. Chem.* **74**, 427.
—— and Moeller (1928). *J. Biol. Chem.* **79**, 429.
Kingston and Schryver (1924). *Biochem. J.* **18**, 1070.
Kleinschmidt (1907). *Z. physiol. Chem.* **54**, 110.
Onslow (1930). Plant biochemistry. (Cambridge.)
Osborne, Leavenworth and Brautlecht (1908). *Amer. J. Physiol.* **23**, 180.
Plimmer (1916). *Biochem. J.* **10**, 115.
—— (1917). Chemical constitution of the proteins, p. 97. (Longmans, Green and Co., London.)
—— and Rosedale (1925, 1). *Biochem. J.* **19**, 1004.
—— —— (1925, 2). *Biochem. J.* **19**, 1021.
Thimann (1930). *Biochem. J.* **24**, 368.
Wada (1930). *Biochem. Z.* **224**, 420.

CCXXIX. VITAMIN A AND CAROTENE.

VIII. HIGH POTENCY VITAMIN A CONCENTRATES.

By THOMAS MOORE.

From the Dunn Nutritional Laboratory, Cambridge.

(*Received November 2nd, 1931.*)

In a previous communication [Moore, 1931, 1] experiments were described which involved the feeding of large amounts of carotene, as red palm oil, to albino rats. Since by this means the vitamin A content of the untreated liver oils could often be made to exceed that of high quality cod-liver oil concentrates it was realised that a good opportunity was afforded to carry the concentration of the vitamin beyond the limits previously attainable.

Preliminary results, communicated at a meeting of the British Association [Moore, 1931, 2], indicated that activities represented by a minimal rat dose of about 0·001 mg., or by colour values 10 times that of carotene, could be attained in concentrates prepared from the liver oils of either rats or pigs which had received liberal amounts of red palm oil. Moreover since oils of widely divergent potency were found to give final concentrates having colour values approaching a common maximum it was considered that these preparations must consist substantially of the vitamin, or alternatively that the vitamin must be associated with other substances in remarkably constant proportion.

In apparent conflict with the view expressed above Karrer, Klussmann and Euler [1931] reported the preparation of a concentrate from the liver oil of *Hippoglossus hippoglossus*, which was at first claimed to display chromogenic activity no less than 130 times that of carotene, equivalent to 10,000 "C.L.O. units." Although a correction has now been made to adjust the ratio from 130 to 13 when pure β-carotene is taken as the standard [Karrer, Morf and Schöpp, 1931; Euler and Karrer, 1931] it still might appear, from the extremely high value given in C.L.O. units, that this concentrate was considerably more potent than those prepared by the present writer[1]. To throw further light on this point Prof. von Euler kindly agreed to supply samples of highly potent fish-liver oils, in order that concentrates derived from them might be compared directly with those derived from rat- and pig-liver oils. The results to be described tend to indicate that the fish oils, rat- and pig-liver oils all yield

[1] Karrer, Morf and Schopp [1931] find that geronic acid is a common decomposition product of carotene and vitamin A concentrates when treated with ozone. From the amount of geronic acid semicarbazone derived from the *Hippoglossus* concentrate they calculate that 50–80 % of actual vitamin A was present.

preparations of approximately equal activity when the same processes of concentration are applied.

Experimental.

Rat-liver oils. Samples of rat-liver oil were obtained from two single animals and from a group of 34 animals. In all cases 15 % of red palm oil was administered for some months before killing, but apart from this essential feature the diets given differed in detail. Thus sample 1 was obtained from 34 rats which received a diet composed of skimmed milk powder 20 %, rice starch 60 %, red palm oil 15 %, salts 5 %; while sample 2 was derived from a single rat which received "light white casein" 20 %, rice starch 60 %, red palm oil 15 %, dried yeast (additional) 15 %. Sample 3 differed decidedly in being obtained from an animal which was exposed to deficiency of the vitamin B complex in connection with another experiment [Dann and Moore, 1931]. The liver oils were obtained by the method previously described, involving digestion of the tissues by 5 % aqueous potassium hydroxide followed by extraction with ether.

Pig-liver oils. The use of pigs was made possible through the kindness of Lever Brothers, Ltd., who undertook the feeding of the animals and also arranged for the extraction of the liver oils. The red palm oil was given as an addendum to the normal diets of the pigs in amounts up to 12 ozs. per pig per day, the feeding being commenced at the "porker" stage (live weight approximately 40 kg.) and continued until the animals reached maturity as bacon pigs. To extract the oils the livers were steamed, minced, pressed free from water, dried and solvent-extracted.

Fish-liver oils. Of the oils supplied by Prof. von Euler one was a sole-liver oil and the remaining two turbot-liver oils. The blue values determined by Miss Rydbom before dispatch were given as 347, 930 and 1200 C.L.O. units respectively.

Concentration of the vitamin.

The methods employed in the concentration of the vitamin were the same in all cases and involved no novel feature. The oils were saponified by a brief boiling with alcoholic potassium hydroxide, ether being used for the extraction of the unsaponifiable matter. The vitamin fraction was usually separated from the bulk of crystalline material by extraction with cold methyl alcohol, the last traces of sterols were then removed by digitonin. The final concentrates were compared by the antimony trichloride method applied in the way previously described. The results obtained are shown in Table I. It should be noted that the values given for the percentage of unsaponifiable matter and "final concentrate" are those actually observed, no allowance having been made for possible losses in manipulation.

$SbCl_3$ absorption band.

Observations by means of a pocket spectroscope revealed the prominent absorption band at 620$m\mu$ typical of vitamin A concentrates.

Biological tests.

As a biological check upon the colorimetric data rat growth tests were carried out in the case of the concentrate from the rat-liver oil No. 2. The basal diet used consisted of Glaxo caseinogen 20 %, rice starch 75 %, salts 5 %, dried yeast (additional) 7·5 %, radiostol (vitamin D) 1 drop per rat daily. The test

Table I.

Description and source	B.U. per g. liver	B.U. per mg. untreated oil	% total unsap. matter	% final concentrate	B.U. per mg. final concentrate	% deviation from average
Rat-liver oil No. 1 from 34 rats	1,600	90	16	3·5*	2100	12
Rat-liver oil No. 2	7,000	450	24	18·2	1900	21
Rat-liver oil No. 3 from "B free" rat	13,000	1000	47	32·6	2300	4
Pig-liver oil No. 1	—	70	17	2·8	2100	12
Pig-liver oil No. 2	—	90	13	3·3	2600	8
Sole-liver oil	—	100	7	3·3	2700	12
Turbot-liver oil No. 1	—	400	14	9·3	3000	25
Turbot-liver oil No. 2	—	450	17·5	13·7	2400	0
				Average ...	2400	

* Digitonin process omitted.

Note. Values given in B.U. per mg. can be converted to the scale of blue values advised by the Pharmacopœia Commission by multiplying by 18·2.

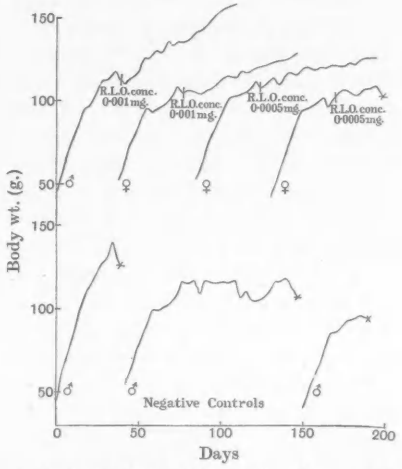


Fig. 1. Biological check of vitamin A activity carried out on concentrate from rat-liver oil (R.L.O.) No. 2.

doses were made up in coconut oil, which possesses a great advantage over many natural oils in not interfering with the antimony trichloride reaction when it is desired to check the stability of the solutions [Norris and Church, 1930]. From Fig. 1 it will be seen that slow growth was restored by doses of

0·001 mg. of concentrate. Of two animals receiving 0·0005 mg. one showed very slow growth while the other died.

Discussion.

Constancy of the final concentration. The outstanding feature of the above results lies in the constancy of the vitamin A activity, as judged by the $SbCl_3$ reaction, attained in the various concentrates. Not only did the values given by the rat- and pig-liver oil preparations as a class approximate closely to those given by the fish-liver oil preparations, but in each class oils of widely different potencies gave final concentrates of almost equal activities. As an extreme illustration of this point attention may be directed to rat-liver concentrates Nos. 1 and 3 which from original colour values of 90 and 1000 B.U. per mg. respectively gave concentrates of almost equal activity. The original blue values of any oil, indeed, could be taken as an indication of the quantity of concentrate that could be obtained, and not of the final strength of the concentrate[1].

Degree of concentration attained. The average $SbCl_3$ value of 2400 B.U. per mg. (Pharmacopœia blue value 45,000) given by the concentrates was about 12 times that given by the specimen of carotene recently supplied for experiments on the international standardisation of vitamin A and 2400 times greater than the specimen of cod-liver oil supplied on the same occasion. In view of the possibility of errors arising through small modifications in technique, personal factors in matching shades, and above all through variations and misunderstandings in the mode of expression of results, it would perhaps be inadvisable to attempt a comprehensive comparison of these figures with those given by Euler and Karrer for their *Hippoglossus* concentrate. A thorough study of such data as are available, however, suggests that this preparation was of the same order of activity as those at present under investigation, and this conclusion is borne out by the closely concordant results given by the *Hippoglossus* and rat-liver oil concentrates in biological tests. It seems possible, from a consideration both of the colour data and of the additional stages of freezing and adsorption adopted in preparing the *Hippoglossus* concentrate, that an activity some 70 % greater than the average value found in the present experiments was attained. Such a superiority would imply that the simplest explanation of the constant activity of the final concentrates obtained in the present experiments—that they contained a predominant proportion of vitamin A—could not hold. It would become necessary to assume that the vitamin in its most active sources is associated with a remarkably constant proportion of non-chromogenic material possessing similar solubilities.

The conversion of carotene. The above work permits further light to be shed on the phenomena accompanying the conversion of carotene to vitamin A.

[1] This statement must for the present be confined to oils similar to those under investigation. With oils of lower colour value, such as ordinary cod-liver oils, it does not seem possible to attain concentrates of such high activity, at least by the simple methods employed in the present work.

In view of the blue values given by the concentrates it must be implied that conversion is accompanied by at least a 12-fold increase in chromogenic activity. At the same time it is not necessary to infer that these spectroscopically different colorations bear a common relation to biological activity. Thus, although Karrer, Morf and Schöpp [1931] state that their *Hippoglossus* concentrate possessed 10 times the biological activity of carotene, this conclusion is not clear from the evidence presented by Euler and Karrer [1931]. A careful comparison of the biological activity of the most active concentrates against carotene in parallel experiments seems to be demanded, and is now in progress in this laboratory.

Another point of interest lies in the extreme change in the solubility in methyl alcohol from the insoluble carotene to the almost miscible vitamin A. This matter has received previous mention [Moore, 1930] but deserves emphasis in view of the recent work of Angus *et al.* [1931] who find that vitamin D is much more freely soluble in this solvent than the pro-vitamin ergosterol. When it is also recalled that vitamin E is likewise readily soluble in methyl alcohol [Olcott and Mattill, 1931] it becomes apparent that the fat-soluble vitamins as a class are sharply characterised in this respect.

Summary.

Vitamin A concentrates prepared from the liver oils of rats and pigs which had received diets containing large amounts of carotene, as red palm oil, were compared with concentrates derived from turbot- and sole-liver oils. Although the initial blue values of the oils varied widely little difference could be detected in the activities of the final concentrates, which approached an average value of 2400 B.U. per mg. (Pharmacopœia colour value 45,000) in the antimony trichloride test, corresponding to a minimal dose of about 0·001 mg. in rat growth experiments. These results suggest either that the concentrates must have consisted substantially of actual vitamin A, or alternatively that the vitamin must have been associated with other substances of similar solubility properties in remarkably constant proportion.

My thanks are due to Dr L. J. Harris for his valuable criticism. The care of the experimental animals was in the hands of Mr A. W. Davies.

REFERENCES.

Angus, Askew, Bourdillon, Bruce, Callow, Fischmann, Philpot and Webster (1931). *Proc. Roy. Soc. Lond.* B **108**, 340.

Dann and Moore (1931). *Biochem. J.* **25**, 914.

Euler and Karrer (1931). *Helv. Chim. Acta*, **14**, 1040.

Karrer, Klussmann and Euler (1931). *Arkiv. Kem., Min., Geol.* **10**, No. 16.

—— Morf and Schöpp (1931). *Helv. Chim Acta*, **14**, 1036.

Moore (1930). *Biochem. J.* **24**, 692.

—— (1931, 1). *Biochem. J.* **25**, 275.

—— (1931, 2). Report of the British Association.

Norris and Church (1930). *J. Biol. Chem.* **85**, 477.

Olcott and Mattill (1931). *J. Biol. Chem.* **93**, 59.

CCXXX. A STUDY OF THE FACTORS INFLUENCING THE CONCENTRATION OF COMPLEMENT IN THE BLOOD.

By THOMAS WILLIAM BRYANT OSBORN.

From the Department of Biochemistry, Oxford University.

(*Received July 23rd, 1931.*)

The present work was commenced with the idea of determining what changes take place in the development of vitamin A deficiency which renders animals susceptible to bacterial infection. It was felt that this would throw new light on the problem of general immunity, since it presented an easy method of lowering and raising the immunity at will.

Of all known factors in general immunity complement is the one which is most widespread, and, therefore, immediately suggested itself for investigation. It is not possible to say dogmatically that a low blood-complement necessitates a low degree of resistance, as there is not enough evidence to prove this. Much of the evidence up to the present points to this conclusion, however, and many workers in this field seem to assume it [see Cadham, 1926]. Moore [1919] tested this assumption by direct experiment and his results showed that it was true for the animals and conditions he used. Working with a number of guinea-pigs low in complement, he found that, though their specific immunity was not significantly below normal, their general immunity was.

It was decided to determine the complement concentration in the sera from vitamin A-deficient rats at various stages of the deficiency, and to compare this with that of normals. Any differences produced by the administration of cod-liver oil were to be investigated.

Several other problems of a similar nature presented themselves during the course of the work. Indications were found that the age of the animal had an influence, and that the ingestion of food produced a change. Observations on these two points were carried out. Huntermuller [1930] has made the interesting observation that doses of ultra-violet radiation raised the complement in man. A series of observations, therefore, was made on the effect of irradiation by means of a mercury vapour lamp on the blood-complement of rats.

Method of estimating complement.

As it was required to make a large number of estimations on the small amount of blood to be obtained from the tail of a rat any method requiring more than one-tenth of a cc. of serum was excluded. In addition to this, an

attempt was made to avoid the inaccuracy of the "serial tube" methods. After considerable experiment, a modification of Eagle's method [1929] was devised, and the writer has found it to be satisfactory in the course of some three thousand estimations.

Sheep's blood, defibrinated by whipping with glass rods and filtered through muslin soaked in saline, was used as a source of the corpuscles. It could be kept in a cold store for periods not exceeding 3 days. The corpuscles were deposited by centrifuging and were washed 3 times with 20 vols. of normal saline (0·9 % NaCl). They were then shaken with a volume of saline sufficient to make the total volume equal to 10 times the original volume of the blood, thus giving an approximately 5 % suspension of corpuscles, assuming the volume of corpuscles equivalent to about 50 % of original blood. The sensitising agent was glycerinated "anti-sheep corpuscle" serum having 1000 units per cc. (Burroughs and Wellcome).

0·01 cc. of this serum—containing 10 units of complement—was made up to 1 cc. with normal saline, and this was added to 1 cc. of the 5 % suspension of corpuscles.

Before constructing the standard "haemolysis time-complement concentration" curve, it was necessary to measure the complement concentration of a volume of serum by the direct serial tube method. The approximate concentration was determined by adding graded volumes of serum, from 0·0025 to 0·025 cc. in each case made up to 0·05 cc. with saline, to 10 tubes, each containing 0·05 cc. of the suspension of sensitised corpuscles. The tubes were placed in a water-bath at 37° for 15 minutes, and the minimal volume of serum necessary for complete haemolysis was determined. This volume was determined more precisely by repeating the operation, adding volumes of serum covering a smaller range. The liquids were measured with a capillary pipette of 0·05 cc. capacity, graduated into 10 divisions. This was used with a small rubber teat. It was found to be important to empty the pipette at about the same rate each time. The haemolysis mixture was made in agglutination tubes of the Dreyer pattern.

Varying volumes of the serum, whose complement concentration had been determined accurately by the direct serial tube method, were mixed with 0·025 cc. of the 2·5 % suspension of corpuscles in agglutination tubes, and the total volume was made up to 0·2 cc. The time for haemolysis was measured in a thermostat at 37°. The amount of complement used, as calculated from the volume of serum used and the known complement concentration of the serum, was plotted against the time for complete haemolysis (Fig. 1).

In constructing the curve a sample of guinea-pig serum and a sample of human serum were used. In making the estimation on a sample of rat blood, it was the practice to use two or more different volumes of serum, and as the different points fell consistently on the curve, it is clear that the curve is applicable to rat's as well as to human and guinea-pig's sera.

It is the custom to express the concentration of complement as the minimal

volume of serum sufficient to haemolyse a definite volume of corpuscles. A more convenient expression is the reciprocal of this volume. For the purpose of this research, it was decided to adopt, as the unit concentration of complement, that amount which is just sufficient to haemolyse an equal volume of a 2·5 % suspension of sheep's corpuscles, sensitised with 10 units of anti-sheep corpuscle serum, in 15 minutes at 37°. The volume of corpuscle suspension used divided by the minimal volume of serum necessary for complete haemolysis gives the complement concentration of the serum in these empirical units.

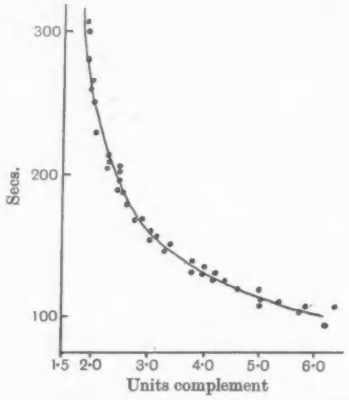


Fig. 1. Standard haemolysis time-complement concentration curve.

In order to estimate the complement concentration of an unknown serum, a volume of this serum is mixed with corpuscles as described, the total volume being made up to 0·2 cc. with normal saline. The time for complete haemolysis at 37° is measured. By reference to the curve, the number of units of complement to give this result is ascertained, and, by multiplication, the number of units in 0·025 cc. of serum—the volume of corpuscles used—may be calculated. This figure represents the complement concentration of the serum.

Certain points of technique require special attention. The end-point of complete haemolysis is rather indefinite. To get as good conditions for the observation as possible, a thermostat with glass sides is used, and a brightly illuminated black and white background is placed behind the tubes.

It was found that, in using these small volumes, any visible roughness of the glass was liable to cause errors, probably due to adsorption effects. It is therefore necessary to select very carefully the tubes to be used. Since any deposit left by tap-water is detrimental to the result, the tubes should be rinsed with distilled water several times before being dried. Both in diluting the serum and in adding the corpuscles, thorough mixing is necessary. This can best be done, in small volumes, by blowing one or two small bubbles into the liquid with the pipette and rocking the tubes so that the bubbles act as mixers.

It will be seen from the curve that the points below 120 seconds are rather scattered. Any haemolysis which takes longer than about 240 seconds has an especially vague end-point. Therefore, for accurate work, only the portion of the curve lying between 120 and 240 seconds should be used.

Rous and Turner [1915] advise particular care in the preparation of the washed corpuscles. Their method of drawing the blood into a weak solution of citrate and gelatin may possess advantages, but, as the method of using whipped blood was proving satisfactory, it was not thought advisable to change it. Brooks [1919] used a buffered Ringer solution instead of saline. He used a bicarbonate buffer, which, of course, has the disadvantage of slowly losing carbon dioxide and so becoming more alkaline. It is difficult to see why he chose to work at p_H 7·8, when, if he were going to use a buffer, he might easily have worked at the p_H of the circulating blood.

An idea of the accuracy of the method may be gained from the closeness of the points to the curve in Fig. 1. It has been found that the error is relatively greater in samples of serum poor in complement. This may be due to the relatively greater amounts of serum-proteins present. An analysis of the last 400 duplicate estimations made by the method, using only that part of the standard curve between 120 and 240 seconds, showed the error to be as follows:

	Below 5 units %	5–15 units %	Above 15 units %
Mean deviation (½ difference of duplicate estimations)	7·6	3·2	2·7
Maximum observed deviation	25	10	7·5

One large source of error was that different samples of sheep's blood differed considerably in the ease with which their corpuscles were haemolysed. This made it necessary to assay each sample. Valley and McAlpine [1928] state that a good way to preserve complement in serum is to keep it away from corpuscles in an atmosphere of carbon dioxide. By preparing a standard sample of serum and keeping it under these conditions, and by titrating each sample of corpuscles against it, a curve for the depreciation of complement in this serum was obtained. A correction was applied to each of those samples of serum which showed a deviation from the curve.

To obtain a sample of blood from the rat, the tail was warmed in normal saline at 45° for 1 minute; the end was cut with a pair of scissors and a few drops were squeezed out on to a waxed plate. A piece of glass tubing had one end drawn out into a capillary. The blood was drawn up into the tube through the capillary portion before it had time to clot, and the end of the capillary was sealed in a flame. When a clot had formed, a fine glass needle was passed between it and the walls of the tube to prevent the clot sticking to the glass. The tube was stoppered and allowed to stand overnight. No appreciable change took place if the sample was kept in a cold store for 48 hours.

Influence of dietary deficiency.

Early experiment. Two groups of animals which were at hand offered an opportunity of testing whether animals on a poor basal diet differed from animals which had taken a liberal supplement of vitamin-containing foods. Rats 420 to 426 had been fed on mixed corn and brown bread from the time of weaning. This is a poor diet, though it is not absolutely deficient in any vitamin. Rats 325 to 328 had received a diet of purified caseinogen, starch and salts, with 3 % cod-liver oil. During part of this time they had been deprived of one of the constituents of the vitamin B complex, but, before the time of the commencement of the experiment, they had been brought to a normal condition by the addition of 6 % marmite to their diet. These animals were put on a diet of corn and brown bread. Another litter of rats was treated in a similar manner. Four, A 18 to A 21, were given corn and brown bread alone, and two, A 17 and A 22, had a liberal supply of green stuff, milk and cod-liver oil added to this diet.

All the animals were given 1 drop of "radiostol" per week throughout the experiment to ensure a good supply of vitamin D. According to the specification of the makers, this is equivalent to about 40 rat units per day, so that even allowing for some depreciation the animals should have been taking an excess of vitamin D.

Each sample was taken in the morning after a fast of 16 hours. The results are given in Table I. It will be seen that the animals which had received a rich

Table I.

A, 3 days after 10 drops of cod-liver oil (0·33 cc.). B, 4 days after 10 drops of cod-liver oil.

Basal diet *plus* excess cod-liver oil, milk and greens		Basal diet. Mixed corn and brown bread	
Rat No.	Complement	Rat No.	Complement
325	15·0	420	4·6
	12·7		6·1
	14·0	421	1·9
	13·0		6·4
326	12·2	422	1·8
	17·1		10·3 A
	14·7	423	4·7
	10·7		8·7
327	10·7		15·6 B
	16·9	424	10·7
328	13·5	425	6·4
	15·4	426	6·6
A 17	7·3	A 18	3·4
	20·8		3·1
A 22	9·0	A 19	3·4
	16·8		3·0
		A 20	3·1
			3·1
		A 21	8·6
			6·2

diet had much more complement than the others. It was significant that rats 422 and 423 had a high complement a few days after a dose of 10 drops of cod-liver oil.

The basal diet seemed to have no absolute deficiency, and it was not clear what it was in the supplements which caused the difference in complement concentrations. It was decided to perform an experiment to compare complement in animals fed on a diet deficient in vitamin A with that in others having cod-liver oil added to the diet. Before this was done it was considered to be advisable to investigate in more detail the influence of age, the ingestion of food and illumination.

The effect of the age of the animal.

In the course of previous investigations indications were found that there was a tendency towards a low complement in young rats. This does not fit in with the findings of workers on human beings [see Goldner, 1929; Gunn, 1914; Moro, 1902]. Kolmer, Matsunani and Trist [1919] state that the blood of young guinea-pigs is more frequently low in complement than that of adults, but give no figures in support of this statement.

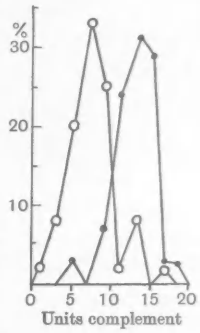


Fig. 2. Percentage distribution of complement values in young and adult rats.
○—○ Young rats.
•—• Normal adult rats.

Complement titrations have been performed on blood from 43 young animals between the age of weaning and 4 weeks from weaning, the animals being weaned between the ages of 3 and 5 weeks according to their weight. Almost all the samples were found to be poor in complement as compared with normal adult animals, whether the samples were taken in the morning after a 16-hour fast, or in the afternoon 3 hours after feeding (Table II and the distribution curves of Fig. 2). In making the comparison, only those figures for young rats are used which are directly comparable with the adult normals, *i.e.* only estimations on blood samples taken in the morning after 16 hours of fasting. The young rats include animals both on a deficient diet and on a rich diet.

The results show that there is a marked tendency for the complement to be low in the blood of young rats and, therefore, only rats of more than 4 weeks from weaning should be used in these experiments.

The effect of ingestion of food.

In the course of the main investigation, indications were received that, in some cases, the ingestion of food caused a change in complement. Cadham [1926], Hadjapoulos and Burbank [1928] and Gunn [1914] mention that a slight rise in complement may be noticed during the day, but they do not correlate this with any digestive activity. Kolmer, Matsunani and Trist [1919]

Table II. *Comparison of complement concentrations in the blood of young and adult rats.*

	Young rats		Normal adult rats	
	No. in range	% in range	No. in range	% in range
0– 2 units	1	2	0	0
2– 4 „	3	8	0	0
4– 6 „	8	20	2	3
6– 8 „	13	33	0	0
8–10 „	10	25	4	7
10–12 „	1	2	14	24
12–14 „	3	8	18	31
14–16 „	0	0	16	29
16–18 „	1	2	2	3
18–20 „	0	0	2	3

	Young rats	Normal adult rats
Percentage below 2 units	2	0
„ „ 4 „	10	0
„ „ 6 „	30	3
„ „ 8 „	63	3
„ „ 10 „	88	10
„ „ 12 „	90	34
„ „ 14 „	98	65
„ „ 16 „	98	94
„ „ 18 „	100	97
„ „ 20 „	100	100

state that blood taken after a 24-hour fast is richer in complement than blood taken immediately after a meal.

A short investigation was made to ascertain whether any definite changes occurred. Out of 23 experiments, 11 showed no change, 12 showed definite changes, comprising a definite fall, a rise and then a fall, or a steady rise (Table III). The basal diet referred to is the fat-free vitamin A-deficient diet described later.

Ehrlich [1906] has suggested that the mechanism for the destruction of bacteria in the blood is the same as that for the utilisation of food material carried by the blood. On this hypothesis, it would be expected that, under certain conditions, a change in some of the agents of immunity might be found during digestion of food. This change might also be expected from a consideration of the suggestion of Walker [1905–06] that the enzymes themselves use a "complement."

Sufficient data have been obtained to show that in some circumstances and in certain animals a change does take place. What the conditions are under which a change occurs, and what regulates the type of change, are problems which will be investigated at a future date.

Effects of irradiation with a mercury vapour lamp.

Huntermuller [1930] investigated the effects of ultra-violet light in winter on 18 human beings, most of whom were normal. His conclusion is that a good, but not excessive, dose causes an immediate slight rise in complement, then a

Table III. *Changes in complement concentration after ingestion of food.*

Rat 289. Basal diet *plus* 10 drops olive oil		Food intake g.	Rat 432. Basal diet		Food intake g.	Remarks
Initial	16·7	—	Initial	16·2	—	No change outside limits
1 hour	16·7	6	1 hour	15·6	8	of accuracy of the method.
2 hours	15·4	5	2 hours	15·8	0	Eleven results of this type
3 hours	15·4	1	3 hours	15·4	0	
4 hours	15·4	2		Total food...	8	
5 hours	16·7	1				
	Total food...	15				
Rat 369. Basal diet			**Rat 331. Basal diet**			
Initial	11·2	—	Initial	13·6	—	Definite immediate fall.
1 hour	10·0	2	1 hour	10·0	3	Five results of this type
2 hours	9·2	2	2 hours	10·6	2	
3½ hours	10·2	2	3 hours	11·6	1	
5 hours	8·3	1	4 hours	12·0	1	
	Total food...	7	5 hours	14·3	0	
				Total food...	7	
Rat 432. Basal diet			**Rat 423. Basal diet**			
Initial	8·7	—	Initial	4·8	—	Rise followed by a fall.
¾ hour	9·7	10	1 hour	13·0	10	Four results of this type
1½ hours	6·9	0	2 hours	5·6	2	
3 hours	6·0	2	3 hours	5·3	0	
	Total food...	12	5 hours	5·3	0	
				Total food...	12	
Rat 327. Basal diet			**Rat 332. Basal diet**			
Initial	14·0	—	Initial	4·0	—	Steady rise.
1 hour	15·4	6	1 hour	4·0	10	Three results of this type
2½ hours	17·0	0	2 hours	11·0	0	
4 hours	19·0	0	3 hours	11·3	3	
	Total food...	6	4 hours	11·5	2	
			5 hours	13·5	0	
				Total food...	15	

fall, and, in 2 or 3 hours, a steady rise reaching a maximum in about 20 hours, then a gradual fall back to the initial value in the course of several days. Unfortunately he quotes no figures to show the extent of the rise, and he does not state whether he finds that it occurs invariably or only in a majority of cases.

In investigating the effects of irradiation on the complement, a mercury vapour lamp was used which produced a definite erythema on human skin at a distance of 6 inches in 2 minutes. The animals were placed in their cages, all food having been removed, at a distance of 20 inches from the lamp. Since the cages had a metal grid bottom, there was no possibility of an effect from the ingestion of irradiated sawdust from the tray of the cage. The experimental animals were controlled by animals, if not from the same litter, of the same age and having an identical feeding history. The controls were kept in cages

apart from the experimental animals, but they were kept under the same conditions during the experiment, being carried into the lamp room with the experimental animals to control any effect of handling or of temperature change. In some cases, a control experiment was performed on the same animal, either before or subsequent to the experimental observation.

Twenty-eight animals were used in this work. Twenty-six experimental and 18 control observations were carried out. In most cases an observation consisted of taking a sample at 10 a.m., immediately before the irradiation, another at 12.30 p.m., and a third at 6 p.m. In a few experiments longer intervals were allowed between sampling. The exposure varied from 2 to 4 minutes at 20 inches.

A typical example of the results obtained is given in Table IV. Of the 26 observations, 6 gave a definite rise, 3 gave a doubtful rise and 17 gave no significant rise above the controls. The important fact, from the point of view of the main thesis of this work, is, that none of the 10 rats on the vitamin A-deficient diet submitted to the light showed a significant rise in complement. The data therefore show that, while the mercury vapour lamp causes a rise in complement in rats on some occasions—a fact which corresponds with Huntermuller's

Table IV. *Examples of the effect of irradiation with a mercury vapour lamp on complement.*

		Complement			
		10 a.m.	12.30 p.m.	6 p.m.	Remarks
Rat A 17	3 mins. irradiation	3·2	8·1	10·9	Rise
Rat A 22	Control	3·8	3·2	4·0	
Rat A 18	3 mins. irradiation	1·8	3·3	3·5	No rise
Rat A 19	" "	1·9	2·8	5·4	? rise
Rat A 20	Control	1·7	1·7	2·9	
Rat A 21	"	1·7	2·3	3·3	
Rat 381	3 mins. irradiation	7·8	10·0	13·1	Rise
Rat 420	" "	14·5	11·4	16·3	No rise
Rat 479	" "	7·8	5·8	10·3	No rise
Rat 421	" "	11·0	9·0	13·4	

observations on human beings—it does not do so invariably. This conclusion is further strengthened by the observation that 8 vitamin A-deficient rats and 7 young rats, which were daily exposed to the radiation for 2 to 3 minutes, all had a complement figure below 8 units on at least one occasion.

Effect of a diet deficient in vitamin A.

The main experiments which follow were controlled by rigidly defining the ages of the animals and the time of estimation of the complement after feeding. Further, the animals were all kept in a darkened room. No rat was used in this experiment under 4 weeks from weaning.

Twenty-four animals from 5 litters were put on a diet of:

	g.
Purified rice starch	70
"Glaxo" vitamin A-free caseinogen	20
McCollum's salt mixture	5
Agar	2
Marmite	6

The marmite was increased to 10 g. during the course of the experiment, as on theoretical grounds 6 % is barely sufficient for the maintenance of an adequate supply of the vitamin B complex. Some of the animals, as controls, were given a supply of the fat-soluble vitamins either as cod-liver oil or as "radiostoleum." All the others were given 1 drop "radiostol" per week to ensure a supply of vitamin D.

As the absence of fats from the diet might have had an effect on the complement level, another group of animals was given

	g.
Rice starch	50
Hardened cotton-seed oil	20
McCollum's salt mixture	5
"Glaxo" vitamin A-free caseinogen	20
Marmite	10

As before, some of these animals were kept as controls and were given 5 drops of cod-liver oil per day, and the others were given 1 drop of "radiostol" per week.

Blood samples were taken from these rats at intervals of about a week. In the beginning and during most of the course of the experiment, the samples were drawn in the morning, after a 16-hour fast. During the latter part of the experiment a few samples were taken at 3.30 p.m., 3 hours after new food had been put in the cages.

In all, 36 animals were used on this part of the work. Twenty-four were placed on the vitamin A-deficient diet, and 12 were given an adequate supply of the vitamin in the form of cod-liver oil or "radiostoleum," and showed no abnormalities in growth or symptoms associated with vitamin A deficiency. An analysis of the figures obtained showed that, while the complement only fell below 10 units in 2 out of 12 normal, it fell below that level in 22 out of the 24 vitamin A-deficient animals.

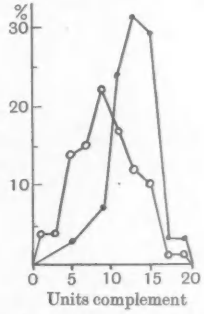


Fig. 3. Percentage distribution of complement values.
•—• Normal rats.
○—○ Deficient rats.

A statistical analysis of the figures is shown graphically in Fig. 3, and in tabular form in Table V. Each point in the first figure represents a reading between the nearest whole numbers. In the second figure the percentages of the total number of readings within certain ranges are plotted for normal and deficient animals. Only readings obtained after a 16-hour fast have been reckoned; those taken at 3 hours after feeding gave a similar result.

Table V. *Distribution of complement estimations for rats on basal diet deficient in vitamin A and the same supplemented with cod-liver oil.*

	Rats on basal diet		Rats on basal diet *plus* cod-liver oil	
	No. in range	% in range	No. in range	% in range
0– 2 units	3	4	0	0
2– 4 ,,	3	4	0	0
4– 6 ,,	11	14	2	3
6– 8 ,,	12	15	0	0
8–10 ,,	18	22	4	7
10–12 ,,	14	17	14	24
12–14 ,,	10	12	18	31
14–16 ,,	8	10	16	29
16–18 ,,	1	1	2	3
18–20 ,,	1	1	2	3

	Rats on basal diet	Rats on basal diet *plus* cod-liver oil
Percentage below 2 units	4	0
,, ,, 4 ,,	8	0
,, ,, 6 ,,	22	3
,, ,, 8 ,,	37	3
,, ,, 10 ,,	59	10
,, ,, 12 ,,	76	34
,, ,, 14 ,,	88	65
,, ,, 16 ,,	98	94
,, ,, 18 ,,	99	97
,, ,, 20 ,,	100	100

It may be concluded, from the results obtained that there is a marked tendency towards low blood-complement in animals deficient in vitamin A or some other factor, other than vitamin D, occurring in liver oils. In the deficient animals marked fluctuations in complement were found which may be due, in part, to the infections to which such deficient animals are susceptible, and, in part, to whatever it is that causes fluctuations from day to day in normal rats and human beings. The fact that animals which are well supplied with liver oil are occasionally deficient in complement, indicates that the presence of liver oils in the diet is not the only condition which must be fulfilled for the maintenance of a high complement. It has been a common experience in bacteriological laboratories that the complement of guinea-pig's serum, high in the summer, may be low in the winter without any alteration in the diet. It is significant that the few instances in which a low blood-complement has been found in rats receiving cod-liver oil have all occurred in winter months.

Discussion.

Irregularity of results. One of the most noticeable features of the results recorded in this work, and, indeed, of any other work which has been done involving the determination of haemolytic complement, is the irregularity of the results. This must be due to the existence of factors influencing complement which have not yet been discovered. This variability makes it necessary

to obtain a large number of observations, and to describe any changes as "tendencies" instead of as definite changes.

In considering these results it should be borne in mind that weight curves of rats deprived of vitamin A are seldom even, and that there is very little constancy about the reaction of deficient animals to cod-liver oil. The day-to-day variation of complement in human beings and rats is difficult to explain. Some of the reported variations have undoubtedly been experimental artefacts due to a variation in the corpuscles used in the haemolysis system. Even when a correction is made for this by the method described for assaying the susceptibility of corpuscles, there is still a large variation.

Relation to other deficiencies. Although lowered resistance is not as prominent a symptom of vitamin B, C, or D deficiency as it is of vitamin A deficiency, there is good support for the view that it is found in these conditions. Werkman, Nelson and Fulmer [1924] showed that guinea-pigs suffering from scurvy were less resistant to bacterial infection than normals, and Findlay [1925] confirmed this. Werkman [1923] showed that rats and pigeons lacking vitamin B were less immune to attacks from *B. anthracis* and the *Pneumococcus* than normal animals. Findlay and Maclean [1925] found that blood from animals deficient in vitamin B or vitamin D had a lowered bactericidal power. Smith and Wason [1923] confirmed this for vitamin D-deficient animals.

The question of whether the lowering of complement in animals fed on a diet deficient in vitamin A is specific, or whether it is common to all vitamin deficiencies, has had some attention. In a cursory investigation, consisting of 8 observations on the blood of 4 rats deficient in vitamin B_1, the author found no significant change in complement. This, however, should by no means be accepted as conclusive. Zilva [1919] found no change in guinea-pigs on a scorbutic diet. Koch and Smith [1924], on the other hand, found a slight rise during the development of scurvy. Smith and Wason [1923] found no change in rats fed on a rachitic diet.

Inanition due to uncomplicated starvation is generally thought to lower resistance to bacterial infection. Koch and Smith [1924] found a drop in complementary power during starvation, but Zilva [1919] found no loss of complement in guinea-pigs after they had been kept on a quantitatively restricted diet for 6 months.

It is certain that there is a lowered resistance in dietary disturbances other than vitamin A deficiency. The evidence is against these being due to diminished complement activity, but this should not be considered as proven.

The action of ultra-violet radiation. The action of ultra-violet light on the body is a much disputed subject. Many people have claimed that it raises the resistance of the body to infection. Colebrook, Eidenow and Hill [1924] showed that it raised the bactericidal power of the blood. This increase was of such a transitory nature that many people were unwilling to attach much importance to it. Colebrook [1929] undertook an extended research and failed to find that ultra-violet light improved the general health in any way, while Hill and Lawrie

[1931] reaffirmed the claim of many that it did. The increased resistance produced by ultra-violet light is thought to be quite apart from its well-known power of producing vitamin D.

The finding of Huntermuller, that ultra-violet light increases complement, is a big advance and may be the means by which many of the actions of ultra-violet light will be explained. The finding that this increase does not occur in all cases of laboratory animals does not detract from the importance of Huntermuller's discovery. These exceptions are of interest in this research because they make it unlikely that ultra-violet light and cod-liver oil cause a rise in complement through the same agency. The evidence for this is that none of the vitamin A-deficient animals submitted to ultra-violet light reacted with a definite rise in complement, and a number of animals which were daily exposed to irradiation had low complements during periods of vitamin A deficiency.

Summary.

1. A method of estimating haemolytic complement is described, which has greater accuracy and uses smaller quantities of blood than the serial tube method now in general use.

2. A unit of complement is defined, which is the reciprocal of the minimal volume of serum sufficient to haemolyse a given volume of sensitised blood corpuscle suspension.

3. Young weaned rats tend to have a lower complement than adult animals.

4. The ingestion of food may cause a variation in the blood-complement of rats.

5. Irradiation with a mercury vapour lamp sometimes causes a rise in complement (Huntermuller), but enough evidence has been obtained to show that this effect is not general.

6. The haemolytic complement tends to be lower in the blood of rats fed on a diet deficient in vitamin A than in the blood of controls receiving cod-liver oil.

The writer is indebted to Prof. Peters for his interest and advice during this research, to Dr V. Reader for many suggestions in regard to the animal experiments, to Dr R. A. Fisher for a statistical examination of the results, to Dr K. H. Coward for advice in vitamin work and to Miss Kempson for her care of the animals. The research was carried out while the writer was the holder of a Rhodes scholarship.

REFERENCES.

Brooks (1919). *J. Med. Res.* **41**, 399.
Cadham (1926). *Can. Med. Assoc. J.* **16**, 352.
Colebrook (1929). *Med. Res. Council Reports*, No. 131.
—— Eidenow and Hill (1924). *Brit. J. Exp. Path.* **5**, 54.
Eagle (1929). *J. Gen. Physiol.* **12**, 821.
Ehrlich (1906). Studies in immunity. (John Wiley and Sons, New York.)
Findlay (1925). *Brit. J. Exp. Path.* **6**, 16.
—— and Maclean (1925). *Biochem. J.* **16**, 574.
Goldner (1929). *Deutsch. med. Woch.* **55**, 390.
Gunn (1914). *J. Path. Bact.* **19**, 155.
Hadjapoulos and Burbank (1928). *J. Lab. Clin. Med.* **14**, 131.
Hill and Lawrie (1931). *Lancet*, i, 182.
Huntermuller (1930). *Strahlentherapie*, **25**, 489.
Koch and Smith (1924). *Proc. Soc. Exp. Biol. Med.* **21**, 366.
Kolmer, Matsunani and Trist (1919). *Amer. J. Syph.* **3**, 407.
Moore (1919). *J. Immunol.* **4**, 425.
Moro (1902). *Jahrb. Kinderheilk.* **55**, 396.
Rous and Turner (1915). *Proc. Soc. Exp. Biol. Med.* **12**, 122.
Smith and Wason (1923). *J. Immunol.* **8**, 195.
Valley and McAlpine (1928). *J. Immunol.* **15**, 313.
Walker (1905–06). *J. Physiol.* **33**, *Proc.* xxi.
Werkman (1923). *J. Inf. Dis.* **32**, 247, 255, 263.
—— Nelson and Fulmer (1924). *J. Inf. Dis.* **34**, 447.
Zilva (1919). *Biochem. J.* **13**, 172.

CCXXXI. ON THE FUNCTION OF TORULIN. AN *IN VITRO* EFFECT OF ANTINEURITIC VITAMIN CONCENTRATES.

By NICOLAI GAVRILESCU and RUDOLPH ALBERT PETERS.

From the Department of Biochemistry, Oxford.

(*Received October 31st, 1931.*)

Recently, the authors [1931, 1] have shown that there is a lowered oxygen uptake[1] as compared with the normal in pieces of minced avitaminous pigeon's brain tissue suspended in glucose-phosphate solution[2]. The change was most marked in the optic lobes. Cure of the pigeon was accompanied by an increase in oxygen uptake, at a time when no increase in body weight occurred. This was proof that a change had occurred in the tissue as the result of introducing vitamin into the body, but not that the increase was due to the addition of vitamin as such to the tissue. Two things suggest that the changes in avitaminosis were due to the local lack of vitamin: (1) that the changes in the brain tended to be localised, and (2) that the most rapid cures of head retraction observed in this Department were induced by the injection of torulin into the cranium; in some cases indeed cure of symptoms has occurred in 30 minutes. Striking as this evidence is, it is not conclusive. The missing entity in the tissue, indicated by the localised chemical lesion, might be some hormone either made from the vitamin and brought to the brain in the circulation, or secreted as the result of a stimulus of vitamin. It has in fact been stated by Cramer [1930] that vitamin B extracts cause a rapid liberation of adrenaline from the suprarenal gland. Such an explanation might also apply to the injection experiments. There are two ways of settling the question; either it might be proved that there is less vitamin B_1 in the optic lobes, *etc.* than normal, which is not practical with the pigeon test owing to the small amounts of tissue involved,

[1] We prefer to use the expression "oxygen uptake" which exactly expresses the observations and is at present non-committal as to their meaning.

[2] About the time that the earlier results were communicated to the Biochemical Society, a paper appeared by Abderhalden and Vlassopoulos [1931] which we did not see until recently. The authors claim to have established a lowering of oxygen uptake in liver, brain and muscle, restored when the birds are dosed with yeast extracts. We believe that the experiments are open to certain objections in the case of the brain tissue which is the only one upon which we have worked. Rather low figures are obtained, very likely owing to the large pieces of tissue used (0·5 g.). Our own preliminary results previously reported, show not much difference in Ringer phosphate solution for oxygen uptake of cerebrum. No attempt seems to have been made to differentiate between different parts of the brain.

or it might be shown that the oxygen uptake *in vitro* is increased by the addition of vitamin concentrates. We believe that this proof has now been obtained, and present the evidence for it below [1931, 2]. If confirmed by further work, it will be the first instance in which an effect of a vitamin has been demonstrated upon a tissue *in vitro*.

Experimental.

The tissue. In all cases we have used birds in the standard condition of opisthotonus induced by our usual methods. Sometimes the cases had been used previously for vitamin tests. We noticed no essential difference between birds which were fresh avitaminous cases and those which had a renewed attack after previous vitamin dosing. There appears to be no point in recording details of the birds used. The effects of vitamin were observed upon the mixed optic lobes and lower parts of the brain (optic lobes/rest) and the cerebrum respectively in the presence of air and of oxygen. Doses of vitamin ranging from 0·01 to 2·0 pigeon-doses have been used, the most satisfactory amount appearing to be 0·1–0·05 pigeon-dose per 3·0 cc. of fluid, containing 0·08–0·10 g. of tissue. Since animal tissues usually contain amounts of vitamin B_1 of the order of 1–2 pigeon-doses per g. of tissue, the dose appears to be optimal if all adsorbed by the tissue. This is very likely to be the case at p_H 7·4.

The tissue was very finely minced as before with a bone spatula and thoroughly mixed, the mixing being continued before the withdrawal of each sample for a bottle. Samples were placed in the bottles in irregular order to avoid any possible influence of drying. Where possible determinations were made in triplicate, but the amounts of tissue are not large. A total of about 0·9 g. of minced tissue can be obtained from the cerebrum, and about 0·4 g. from the mixed optic lobes/rest. Cerebellum was not included in the study as no change in this tissue was previously observed.

The measurements. The methods used were essentially the same as previously employed; the glucose-phosphate buffered solution was made up as before; the precautions of Dixon and Elliott [1930] as to rate of shaking and absorption of CO_2 were observed as previously. The constants were checked by direct determinations under the conditions of the experiments at 38°. In the first series air was used in the bottles and in the later oxygen. The bottles were evacuated to a pressure of 710 mm. Hg before filling with oxygen and washed out once with the gas. About 20 minutes elapsed from the guillotining of the bird to placing the apparatus in the warm bath when air was used, and about 30 minutes with oxygen. It was our practice to shake for 12 minutes before taking the first reading, and to make subsequent readings every 10 minutes for 60 minutes. The results were calculated as an average for the hour of observation. With the cerebrum, the rate of oxygen uptake remained fairly constant though there was a tendency to "balling" of the tissue in some cases. This was noticeable as a lowered oxygen uptake in the first periods. In some cases allowance could be made for this by a comparison of subsequent periods

(Exps. 99, 114 and 117 have been so corrected). Both in cerebrum and optic lobes/rest, comparison of results with and without vitamin for the last 40 minutes of the periods of shaking gave substantially the same results. With the optic lobes/rest the rate of oxygen uptake fell off throughout the period of observation to an amount which varied in different experiments from 33 to 50 % of the initial rate.

The errors of the observations cannot be computed exactly. Those due to the apparatus alone are not larger than 1 %. As we have worked with a mixed tissue, we have included in the Tables all those results which could not be explained by us as due to experimental error. It appears from the figures that our results are subject in the extreme to variations of the order of $\pm$ 10 %, though many duplicate estimations are much better than this. Doubtless several of the discrepancies are due to undetected experimental error; others are probably due to unequal adsorption of vitamin upon the pieces of tissue.

Vitamin solutions. Torulin (yeast B_1) concentrates of different purity were used. The crude preparation A consisted of a 50 % alcohol concentrate of activity about 3·0 mg./pigeon-dose (1·5 B_1 units) [for details see Carter, Kinnersley and Peters, 1930][1]. Preparation B was less impure, and was an X concentrate, prepared initially from brewer's yeast, of activity 0·05 mg./day. This constitutes a purer source of vitamin B_1 than the Y concentrates, so far as other factors in the B-complex are concerned. Preparation C was one of our purest concentrates of activity 12γ/day [see Kinnersley and Peters, 1930, 2]. The solutions were freshly prepared; two drops of water were added to the alcoholic solution (about 0·1 cc.), the alcohol present was removed by heating on the water-bath with continuous shaking; a trace of sulphuric acid (N/20) was then added, and the heating continued for 2 minutes. This was necessary in most cases in order to remove the last traces of barium with which it is our custom to store many of the preparations. The torulin solution was diluted to about 0·5 cc., a minimal amount of phenol red added, and the whole carefully neutralised to p_H 7·4; water was added to make the vitamin addition 0·1 cc. in each bottle. In all the later experiments, a control tube was treated similarly as to sulphuric acid, phenol red and soda and added to the control brain sample.

In the experiments in which alkali-treated vitamin was used, the procedure was the same up to the alcohol stage. After this, however, the sample was divided into two parts. One of these was neutralised approximately, the requisite amount of NaOH added and the sample heated in the water-bath in a test-tube immersed in the boiling water, with a funnel to prevent excessive concentration. After treatment with NaOH, a similar amount of cold NaOH was added to the other sample of active vitamin; the two were then neutralised in a similar way with addition of the necessary amount of phenol red. In this way the effects of excess of salt were controlled. The additions of torulin were made immediately before placing the apparatus in the bath, to avoid possible inactivation.

[1] 1 pigeon-dose = approx. 15 mg. Jansen acid clay $\equiv$ 1·5 vitamin units.

Results.

The results are given in Table I, and will be considered in detail below. Before doing so mention must be made of the effect of oxygen.

Table I. *Protocols. Oxygen uptake, mm.³/g./hr.*

Air. Avitaminous birds at H.R. stage.

(*a*) Control and vitamin.

	Cerebrum				Optic lobe and rest (mixed)			
Exp.	Control	Vitamin	Dose added	Diff. % average	Control	Vitamin	Dose added	Diff. % average
84	1230 1190	1245 1225	2·0 A	+2	—	—	—	—
99	1100 1090	1090 1140	0·1 A	+2	790 950 940	880 940 1050	0·1 A	+7
100	—	—	—	—	810 880 960	840 1015 1030	0·1 A	+7
106	925 —	1290 1270	0·1 A	—	930 930	1120 1050	0·06 A	+16
107	1155 1220 1285	1190 1240 1380	0·06 A	+4	870 940	890 1060	0·06 A	+8
108	1080 1130 1130	1150 1180 1240	0·1 A	+7	—	1130 980	0·7 A	—
109	1080 1125 1180	1140 1290 1350	0·1 A	+11	810 880	950 1070	0·1 A	+20
110	840 —	1090 —	0·1 A	—	745 765 795	835 890 900	0·1 A	+13

(*b*) Control, alkali-treated and active vitamin.

	(1)	(2)	(3)		Diff. %		(1)				Diff. %	
Exp.	Control	Alkalised vitamin	Active vitamin	P.D.	1/2	2/3	Control	Alkalised vitamin	Active vitamin	P.D.	1/2	2/3
112	1080 1120	1070 1110 1130	—	0·1 A	Nil	—	870 920	855 950	—	0·1 A	+1	—
114	1030 1170	910 1080	1214 —	0·1 B	−10	—	—	760 815	850 865	0·1 B	—	+9

In presence of oxygen.

(*c*) Comparison of oxygen and air, together with vitamin and oxygen.

	Cerebrum					Optic lobes and rest (mixed)				
Exp.	Air	(ii) O_2	(iii) O_2+vit.	Dose	ii/iii Diff. %	Air	O_2	O_2+vit.	Dose	ii/iii Diff. %
117	980 995	1220 —	1470 —	0·1 A	[+19]	810	780 920	930 1000	0·1 A	+15
118	955 1015	1330 1360	1520 1580	0·1 A	+15	810	905 965	920 1150	0·1 A	+10
119	1070 1150	1470 1550	1645 1700	0·1 A	+10	1135	1040 1190	1240 1275	0·1 A	+13
120	890 975	1250 1320	1220 1470	0·09 B	+5	1030	1140 1170	1330 1460	0·08 B	+21
123	1120 1130	1200 1500	1480 1620	0·11 B	+15	805	1020 1030	—	—	—

Table I (*cont.*)

(*d*) Active, alkali-treated vitamin and controls in presence of oxygen.

	Cerebrum						Optic lobes and rest (mixed)						
	(1)	(2)	(3)		Diff. %		(1)	(2)	(3)		Diff. %		
Exp.	Control	Alkalised vitamin	Active vitamin	Dose P.D.	1/2	2/3	Control	Alkalised vitamin	Active vitamin	Dose	1/2	2/3	Remarks
124	—	1530 1720 1810	1600 1660 1870	0·1 B	—	+2	—	745 972	1090 1130	0·1 B	—	+28	2 *N* NaOH 20 min.
125	—	1330 1495 1660	1720 1730 1750	0·07 B	—	+16	—	1005 1180	1030 1250	0·07 B	—	+5	0·5 *N* NaOH 45 min.
126	1130 1210 1280	—	1190 1220 1325	0·01 B	+2	—	752 760	—	810 915	0·01 B	+13	—	
127	—	1470 1790	1500 1750 1790	0·05 B	—	+3	—	1210 1350	1340 —	0·05 B	—	Nil	0·5 *N* NaOH 30 min.
128	—	1610 1670 1690	1690 1720 1820	0·05 B	—	+5	—	1130 1260	995 1210	0·01 B	−8	—	0·5 *N* NaOH 30 min.

Active, alkali-treated vitamin, *etc.* (*continued*).

	Cerebrum						Optic lobes and rest (mixed)						
	(1)	(2)	(3)		Diff. %		(1)	(2)	(3)		Diff. %		
Exp.	Control	Alkalised vitamin	Active vitamin	Dose P.D.	1/3	2/3	Control	Alkalised vitamin	Active vitamin	Dose P.D.	1/3	2/3	Remarks
129	—	1510 1550 1580	1340 1350 1720	0·05 B	—	−9	—	1070 1045	1345 1380	0·05 B	—	+30	0·5 *N* NaOH 30 min.
130	1705 1825	1710 1760	1920 1935	0·01 B	+10	+11	—	1120 1160	1080 1160	0·01 B	—	+2	0·5 *N* NaOH 30 min.
131	1175 1185	1330 1400	1455 1500	0·05 B	+25	+7	—	925 930	980 1280	0·05 B	—	+23	2 *N* NaOH 60 min.
133	1190 1270	1390 1420	1440 1490	0·10 B	+19	+4	—	890 915	1040 1070	0·10 B	—	+20	2 *N* NaOH 60 min.
138	1550 —	— —	1560 1550	0·1 C	—	Nil	1125 1180	—	980 1135	0·01 C	−10	—	
139	1340 —	— —	1410 1460	0·1 B	+7	—	1030 895	—	875 1110	0·01 B	+3	—	
150	1200 1240	—	1300 1380 1425	0·10 B	+17	—	910 940	—	900 975	0·05 B	+1	—	
151	1410 1490 1510	—	1490 1520 1600	0·10 C	+14	—	805 920	—	970 1140	0·1 C	+16	—	
153	1280 1380	1190 1220	1220 1260	0·05 C	−7	−4	—	1240 1330	1390 1570	0·05 C	—	+15	2 *N* NaOH 30 min.
154	1640 1700	1500 1560	1530 —	0·05 C	−9	Nil	—	1170 1280	1400 1400	0·05 C	—	+14	2 *N* NaOH 30 min.
164	—	—	—	—	—	—	1150 1225	—	1262 1304 1340	0·1 C	+10	—	Brains of 2 birds mixed
165	—	—	—	—	—	—	1120 1120 1190 1175	—	1204 1340 1220 1200	0·1 C	+6	—	Brains of 2 birds mixed
184	1302 1430	—	1240 1425	0·1 C	−25	—	1202 1225	—	1280 1382	0·1 C	+10	—	
185	—	—	—	0·1 C	—	—	990 1130 1152	—	1240 1310	0·1 C	+16	—	

Comparison of the results with oxygen and air respectively.

It was stated previously that the uptake of oxygen could be increased by performing the experiments with oxygen in the bottle instead of air, but that our general conclusions were not influenced by this. In Table II we have recorded the average results from several recent experiments together with those from our previous paper. It will be seen that the new ones are quite in agreement with the old. The conclusion that there is lowered oxygen uptake in glucose-phosphate solutions in the avitaminous brains is made more certain by these results. The increase in oxygen uptake for oxygen as against air with

Table II. *Comparison of oxygen uptake in presence of air and oxygen.*

(*a*) Different birds. mm.³/g./hour*.

Tissue		Air	Limits	Oxygen	Limits	Increase
Cerebrum	N.	**1315**	(1000–1600)	**1810**	(1470–2220)	+38 %
	Av.	**1030**	(880–1210)	**1341**	(980–1670)	+32 %
Optic lobes/rest	N.	**1100**	(960–1280)	**1480**	(1300–1600)	+38 %
	Av.	**880**	(805–1135)	**1055**	(980–1670)	+16 %

N. = Normal. Av. = Avitaminous.

(*b*) Comparisons upon tissue from same bird (avitaminous).

	Cerebrum		OL/LP	
	Air	O_2	Air	O_2
(1)	990	1220	810	850
(2)	980	1340	810	930
(3)	1110	1510	1135	1120
(4)	880	1280	1030	1150
(5)	1050	1350	805	1030

* 1000 mm.³/g./hr. = Q_{O_2} (Warburg) 5·0 approx.

normal brains is rather variable, amounting on the average to + 38 % for cerebrum and + 32 % for the rest. This would be interpreted easily upon the usual view that the presence of oxygen activated underlying pieces of the tissue not otherwise reached at the pressure of oxygen in air, if it were not for the results with the avitaminous tissue. With the avitaminous cerebrum, increased oxygen pressure increased the uptake by 30 %. With the avitaminous mixed optic lobes and lower parts, however, the uptake was not increased on the average more than some 20 %, and in several cases which are given in abstract in Table II a comparison of the tissue with and without oxygen shows no difference. Since there is no apparent difference in the state of division of the tissue for normal and avitaminous brains, doubt is thrown upon the simple explanation that the increases observed in these minced brain samples are due merely to increased penetration of oxygen. As previously mentioned, at the conclusion of the experiment the tissue is mostly in the form of a fine suspension. The matter merits further attention. In the series of experiments which follow, the effects of vitamin concentrates have been followed both in the presence of air and of oxygen; rather similar results have been obtained with each.

Effects of vitamin concentrates.

In the protocols given in Table I, we have given the experiments as they were obtained, and have included all those in which no obvious experimental error could be detected. In this way the whole evidence upon which conclusions are based is before the reader. It will be recalled that previously we reached the conclusion that there are differences between the cerebrum and the rest of the brain in avitaminosis. Kinnersley and Peters [1930, 1] advanced the view that the changes in lactic acid metabolism commenced in the lower parts of the brain and gradually spread to the cerebrum, and in our previous paper we concluded that the two parts of the brain were not always equally affected. In this work too, we have found marked differences between the behaviour of the two parts, and are therefore considering them below as separate tissues.

Mixed optic lobes and the rest.

A casual glance at the protocols for this part of the work shows that in spite of variations the general trend of the results is towards an increase in the presence of vitamin. The results are shown diagrammatically in Fig. 1. Out of 19 experiments, 6 in air, and 13 in oxygen, 10 show a clear increase for the vitamin-treated tissue in the sense that there is no overlap in the figures; 7 of these 10 are in the presence of oxygen[1]. We have examined and excluded the possibility that some particular use of the same bottles for the same parts of the experiment might have led to the result, as also that the order in which the tissue was transferred to the bottles might produce the effects found. The other possibility that the results might be the consequence of the method of determining constants has also been eliminated by finding these directly at 38°, which led to a slight constant correction. The problem therefore narrows down to the following question; what are the chances that we have made favourable selections in these 10 experiments? To take a concrete case, Exp. 119, we might suppose that the possible errors in sampling this particular tissue lay between 1000 and 1300, and that by chance we had selected our samples to give the apparent increase for vitamin. The matter is one of statistics, and the probability against this favourable selection is very great. The mean percentage difference is 10 % approx. It can be calculated that a mean percentage difference of 4·8 % would be likely to occur by chance only once in 100 times. We feel therefore that the results constitute proof that the addition of vitamin concentrates increases the oxygen uptake. The effect is catalytic: where the preparations B and C have been used, a larger weight of oxygen is taken up by the vitamin-treated tissue than the weight of solid added. As an example with the purest C, 0·5γ added to 3·0 cc. of the solution increased the average O_2 uptake by 15γ oxygen. Controls showed that no increase took place in absence

[1] The remainder show an average increase except Exp. 138, which cannot be explained and was performed with 1/100 pigeon-dose of vitamin, a rather small amount.

of tissue. The average increase of 12 % is about half that of the lowered oxygen uptake previously recorded.

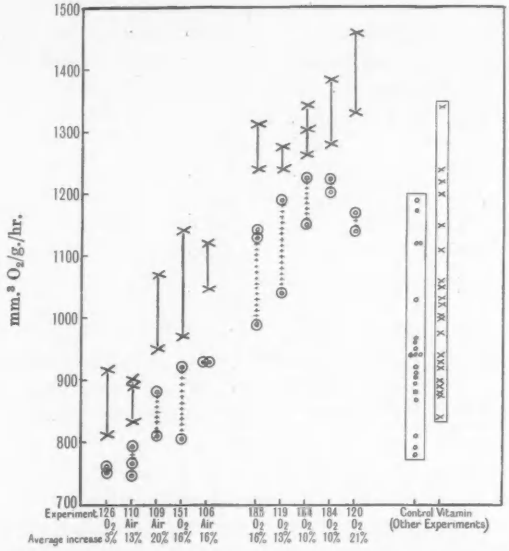


Fig. 1. Chart showing results for mixed optic lobes/rest with and without vitamin. × + vitamin concentrates, O control. Ordinates O_2 mm.³/g./hr. Experiments arranged along abscissae in increasing order of basal oxygen uptake. Other results for Exps. 99, 100, 107, 117, 118, 150, 165, 139, *etc.*, grouped together and shown on right of chart.

The next question, whether the increase is due to the vitamin itself or to some other base in the concentrates, cannot be settled absolutely in the absence of pure vitamin B_1. Concentrates of quite different purity give the same result. Treatment with alkali is known to inactivate vitamin B_1. It has been shown previously that 1 hour's heating at the temperature of the boiling water-bath with 0·5 *N* NaOH will inactivate a concentrate of 0·06 mg. activity. This probably varies for the preparation, and requires reinvestigation [Kinnersley and Peters, 1928]. In the experiments below, it appeared from pigeon tests that 30 minutes' heating at the temperature of the boiling water-bath with approx. 0·5 *N* NaOH was not enough to inactivate preparation B, but that 30 minutes with 2*N* NaOH was enough. We have compared the effect of alkali-treated concentrates with untreated controls, containing a similar amount of salt. In Table III the results of this treatment have been collected for the oxygen experiments and arranged in order of alkali treatment. 0·5 *N* NaOH for 30 minutes seems to be marginal, whereas 2*N* NaOH for 30 minutes seems to be sufficient to inactivate the factor concerned. Such a treatment was found by animal tests to reduce the activity to less than 10 % of the original. The

Table III. *Oxygen uptake (mm.³/g./hr.) for mixed optic lobes/rest with active and alkali-treated vitamin B concentrates.*

Exp. No.	Dose added	Preparation	Vitamin: Alkali-treated	Vitamin: Active	Difference	Alkali treatment at 100° approx.
127	0·05	B	1210 1350	1340 —	[? nil]	0·5 *N* 30 min.
129	0·05	B	1070 1045	1345 1380	+30 %	0·5 *N* 30 „
125	0·07	B	1005 1180	1030 1250	+5 %	0·5 *N* 45 „
124	0·10	B	745 972	1090 1130	+28 %	2*N* 20 „
153	0·05	C	1240 1330	1390 1570	+15 %	2*N* 30 „
154	0·05	C	1170 1280	1400 1400	+14 %	2*N* 30 „
131	0·05	B	925 930	980 1280	[+23 %]	2*N* 60 „
133	0·10	B	890 915	1040 1070	+20 %	2*N* 60 „

Note. The above experiments in oxygen. Exp. 114 (air) gives a similar result. Exps. 128 and 130 not included, in which heating was for 30 min. with 0·5 *N* NaOH, and only 1/100 dose used.

inactivating effect of alkali therefore is roughly the same upon vitamin B_1 and the factor considered. Here again 0·01 pigeon-dose seems to be marginal. Taken together with the fact that our purest concentrates show the effect, we have reached the best proof at present available that the oxidation factor concerned is vitamin B_1. The necessary concentration so far as can be judged from this limited series of experiments lies between 0·1 and 0·01 pigeon-dose, the latter being marginal. With preparation C, this means a dilution in 3·0 cc. of about 1 in 30,000,000.

The cerebrum.

We have mentioned previously our conviction that the condition in this tissue is different. To the eye, the minces from the two tissues appear quite dissimilar. It has been steadily forced upon us during the course of this work that the behaviour towards vitamin concentrates is also different, a conclusion quite at variance with our preconceptions. The effects of different concentrates do not appear to be identical, nor does the inactivation follow the same course, though there is ample proof that the addition of certain concentrates improves the uptake of oxygen.

Out of 6 completed experiments in air, 2 showed no overlap between the figures (Nos. 99 and 108). The remainder only showed a small increase upon the average. For experiments in oxygen, out of 13 completed experiments, 6 (Nos. 118, 119, 130, 131, 133, 150) showed an increase such that there was no overlap in the duplicate observations (in some cases triplicate). Of the remainder, 4 showed increases upon averages of different degree. Three gave a

decrease. This we cannot explain at present, but it is noteworthy that the two significantly negative results were obtained with the purest preparation (90 A). There is a definite tendency (see also 138) for this preparation to be less effective than the others in increasing oxygen uptake in the cerebrum. Before discussing the possible meaning of this, we can consider the results for activated and inactivated vitamin. In the experiments with each bird, the vitamin solution was the same throughout; hence any variations in the inactivation should affect both cerebrum and the rest equally. It is noticeable that there are cases in which the difference for active and inactive vitamin is definite for the optic lobes, *etc.*, but is not so for the cerebrum. This is illustrated by Exps. 124, 129, 133, 153 and 154. It appears to be more difficult to inactivate the preparation for cerebrum than for the rest. From each set of experiments therefore we reach the conclusion that in the cerebrum the lowered oxygen uptake is due to causes other than vitamin B_1 deficiency alone. It is possible that the vitamin is unstable in the presence of factors sometimes associated with the cerebral tissue, but in view of the apparent difficulty of inactivating the tissue for the cerebral factor, a more attractive hypothesis is that the cerebral tissue is suffering from an additional deficiency of some other factor. This would be consistent with the very irregular action of our purest preparation, which for instance contains very little vitamin B_4. Such speculations must be rather random in the absence of work upon the more obvious points which require an answer. Much might be learnt for instance from a comparison of the effect of vitamin upon the tissue in the presence and absence of glucose. This should settle how far the effect was exerted upon the residual oxygen uptake. Our present conclusion must be that vitamin B_1 is less consistently effective in catalysing an increased oxygen uptake in the cerebrum than in the other parts of the brain. This confirms the opinion reached previously that the condition in the two parts of the avitaminous brain is different [see Kinnersley and Peters, 1930, 1] and ourselves [1931, 1, Appendix], and that the optic lobes and lower parts are more directly connected with the vitamin B_1 deficiency as such.

Normal brains and washed tissues.

The behaviour of normal brain tissue and of washed tissues to vitamin concentrates does not influence the particular facts under discussion in this paper, though it might have some bearing upon their interpretation. The few experiments, which we have made up to the present, do not justify us in drawing any definite conclusions.

Discussion.

Analysis of the behaviour of the minced optic lobes and rest of the pigeon's brain may seem a devious way to search for the nature of vitamin B_1 deficiency, and a test for vitamin B_1. We have been led to it by failures to demonstrate the action of vitamin concentrates upon more obvious tissues with lactic acid

systems, following the work upon the lower parts of the brain [Kinnersley and Peters, 1930, 1]. It may be hoped that these experiments will lead to some easier test for vitamin B_1, which may be specific enough to settle some of the present confusion, inevitable as long as we are unable to assess the precise influence of other factors of the complex upon vitamin B_1 tests. It is possible, though we think unlikely in view of what has been said earlier, that these reactivations are not due to the vitamin. The proof that a catalytic effect is involved decides between the three possibilities discussed in our earlier paper. It is not clear of course what oxidase system is involved, but it is hoped that experiments at present in progress will decide this point.

Accepting the view provisionally that the vitamin B_1 is itself concerned, it may be inferred that it takes part as such in tissue metabolism without the intermediary action of an internal secretion; its well-known presence in tissues is therefore not an accident of storage.

So far as we are aware, this appears to be the first experimental support for a direct relation between a local biochemical lesion and an abnormal condition of the central nervous system. The argument can be briefly stated. Injection of torulin into the cranium very rapidly relieves opisthotonus symptoms; addition of the same *in vitro* to the optic lobes/rest of the brain restores in part the lowered power of oxygen uptake present in avitaminosis. With the same part of the brain is associated especially the increase in lactic acid found by Kinnersley and Peters [1930, 1]; vitamin rapidly induces an increased rate of disappearance of lactic acid in the tissues of the avitaminous animal [Fisher, 1931]. Realising that there is still much that is not clear, the lesion seems evidently to affect the tissue metabolism of carbohydrate, a view which has been often mooted. Perhaps for the first time, we obtain here experimental grounds for belief in the great importance of the antineuritic vitamin B_1 as such in the normal functioning of the central nervous system.

Conclusions.

1. The oxygen uptake of finely minced pigeon's brain tissue (glucose-phosphate solution) is increased on an average some 40 % by performing the estimations in oxygen instead of air, in the case of cerebrum and the mixed optic lobes and parts below from the normal brain. With the avitaminous (B_1) brain, less or even no increase takes place with the mixed optic lobes and lower parts.

2. In the presence of oxygen the lowered oxygen uptakes previously observed in the brain in avitaminosis B_1 are more marked.

3. Partial restoration of oxygen uptake *in vitro* is observed upon addition of vitamin B_1 concentrates to the mixed optic lobes and lower parts of the avitaminous pigeon.

4. This action of the concentrates follows the vitamin B_1 and is inactivated by treatment with alkali as is vitamin B_1. The action is catalytic.

5. The effects are not always obtained with the cerebral tissue, which behaves differently.

6. Vitamin B_1 is directly concerned with the oxidative metabolism of the lower parts of the pigeon's brain.

We are indebted to the Government Grant Committee of the Royal Society and to the Medical Research Council for grants towards this work. We are also grateful to Mr H. W. Kinnersley for the concentrates used, to Mr Meiklejohn for help in some of the experiments, and to Mr Yates of the Statistical Department, Harpenden, for aid in the analysis of the figures.

REFERENCES.

Abderhalden and Vlassopoulos (1931). *Pflüger's Arch.* **226**, 808.
Carter, Kinnersley and Peters (1930). *Biochem. J.* **24**, 1832.
Cramer (1930). Personal communication.
Dixon and Elliott (1930). *Biochem. J.* **24**, 820.
Fisher (1931). *Biochem. J.* **25**, 1410.
Gavrilescu and Peters (1931, 1). *Biochem. J.* **25**, 1397.
—— —— (1931, 2). *J. Physiol., Proc. Physiol. Soc.* (In press.)
Kinnersley and Peters (1928). *Biochem. J.* **22**, 419.
—— —— (1930, 1). *Biochem. J.* **24**, 711.
—— —— (1930, 2). *Biochem. J.* **24**, 1856.

CCXXXII. THE ASSIMILATION OF ALUMINIUM BY THE HUMAN SYSTEM.

By SAMUEL JUDD LEWIS.

From 329 High Holborn, London, W.C. 1.

(*Received September 22nd, 1931.*)

The scientific work relating to the assimilation of aluminium by the animal system has for the most part failed to give satisfaction, even when by weight of evidence it was fairly conclusive. The chief reasons for this are that the quantities of aluminium absorbed are very small, frequently much less than one part in a million parts of blood or tissue and rarely appreciably more, while the purest reagents obtainable normally and employed in making the search can very rarely if ever be obtained quite free from the element in question. The weight of aluminium present in the necessarily large mass of reagents is often greater than that in the material to be extracted.

Attempts to simplify this problem by resort to the spectrograph are presented in the following observations.

The first experiments were directed to discovering how much, if any, aluminium is present in normal blood obtained first from the slaughter-house and then from man, and to finding and estimating suitably small quantities of aluminium mixed well into the blood before drying down.

The experimental procedure ultimately adopted was to ash a few grams of blood in a new silica crucible after it had been prepared by treatment with hydrochloric acid followed with hot concentrated sulphuric acid. The blood was gently ignited until free from carbon; then 10 mg. of the ash, representing approximately 1 g. of blood, were transferred to the lower electrode of an arc-lamp and the spectrum observed in the usual way by means of a large Hilger quartz spectrograph.

Under the conditions of experiment the two principal aluminium lines 3961·540 and 3944·032 Å. were well in evidence, although feeble, when the proportion of added aluminium was one part in a million parts of blood. The lines were still definitely discernible when the concentration was only one-fifth of that named, and in the later experiments with one in ten millions they could be recognised with certainty.

The absence of extraneous aluminium was assured by (*a*) cleaning the crucible as above described, (*b*) using no reagent, and (*c*) taking a comparison spectrogram of the electrodes alone immediately before placing thereon the ash, to serve as a blank. There is one necessary condition which may at first

sight appear to contradict these tenets, namely, the moistening of the ash with a few milligrams of dilute acid, but as the electrodes were similarly moistened, the blank (*c*) was complete; no aluminium was or could be discovered in the 3 or 4 mg. of acid used, even if it did contain the amount of aluminium said to be present in ordinarily pure reagent acid.

Under the conditions of experiment, sheep's blood was usually found to contain about 1–2 parts per million aluminium; human blood was almost invariably free, that is, it contained less than one part in five or ten millions, although occasionally a very small quantity was indicated.

A series of trials with blood charged with various known quantities of aluminium, round about one part per million, showed a well-graduated series of intensities corresponding with the quantities of element added and gave full assurance that the quantitative indications were satisfactory within the limits shown in the following Tables.

In order to ascertain whether aluminium is taken up by the blood from food two men were dieted for 24 hours or more with food supposed to be free from aluminium; then each of them partook of a meal containing a definite quantity of aluminium. Just before the meal a sample of blood was taken; 3 hours after the meal a second sample, a third 4½ hours after, and a fourth sample 48 hours after. Each sample of blood was then carefully ashed and submitted to spectrographic examination.

The results were that no aluminium could be detected in the first normal samples of blood, nor indeed in those taken 3 hours after the meal. In the case of one subject no aluminium was found in either of the two succeeding samples, except a minute trace after 48 hours. With the other subject traces were discovered after 4½ hours and rather more after 48 hours, but the quantity was very small, probably not more than one part of aluminium (Al) in five and three million parts of blood respectively.

Taking several specimens of blood from the same subject was very trying; and when it was found that normal human blood contains usually no aluminium, or only a minimal quantity, it was decided to take advantage of blood from patients undergoing operations, feeding them with aluminium-containing food before or after the operation according to the nature of the specimens required. This procedure eliminated the drawing of more than occasional special specimens of blood. Several patients awaiting operation had included in their daily diet for a convenient number of days one meal of scones containing about 94 mg. of aluminium, the rest of the diet being normal. This course had an important advantage in the patient remaining normal in temperament and digestive function during the ingestion of the aluminium. Blood was taken some time before commencing the feeding and 24 hours after the last meal.

Assuming that there are about 6 kg. of blood in the human body, the proportion of aluminium in the blood, supposing the whole of the aluminium passed into the blood-stream, would be about 15 parts per million, but this is

of course impossible of realisation, and all considered, not more than a tenth of this amount is likely to appear in the blood.

The observations on the analysis of blood were of three types: (*a*) normal blood; (*b*) normal blood into which known quantities of aluminium had been mixed before drying down; (*c*) blood from subjects who had been fed on aluminium-containing scones.

Preliminary tests, made during the development of the method, are collated in Table I. In the earlier ones, the limit of sensitivity was about 1 in 3,000,000, but in the later ones, the limit was 1 in more than 10,000,000. "W." signifies a particular hospital.

Table I.

Subject	Aluminium—parts per million		
	In normal blood	Added	Found
W. 1	6 times 0 3 times say 0·1 or 0·2	—	*v. infra*
W. 2	0	—	—
W. 3	0	1	1·0, 0·6, 1·4, 1·0
W. 4	0, 0	1	1·4, 1·0
W. 5	0, 0	3	*v. infra*
W. 6	0	3	*v. infra*
W. 7	0	—	*v. infra*
W. 8	0	3	3·0, 3·0
W. 9	—	3	3·0, 3·0, 3·0, 3·0
W. 10	0	1	1·0
W. 11	0	1	0·6

W. 5 and 6. The method for the aluminised ash was modified, with a view to concentrating the Al, by washing away the soluble portion. The results were unduly low, 1·7 and 1·4 respectively, whence it is assumed that the aluminium is partly in a soluble form, possibly as alkaline aluminate, but this was not studied.

W. 7. 8 g. of this specimen were ashed at full temperature in a closed apparatus, in which the vapours evolved were aspirated through water contained in absorption tubes, to ascertain whether any iron was thus vaporised in the form of ferric chloride. No iron was detectable in the distillate by the usual reagents. From this it was inferred that since iron is not volatilised under the conditions, it is probable that aluminium would not be volatilised.

Blood from two sheep was examined. Numerous preliminary experiments showed that the blood from sheep No. 1 contained about 3 parts of Al per million; that from sheep No. 2 about 1 part.

Results of the principal series of experiments on human subjects are collated in Tables II and III. They demonstrate that aluminium is absorbed by the blood of a subject who eats food containing aluminium, and that the amount absorbed does not increase appreciably by continuing the feeding, unless one case ("L") is to be regarded as representative of a certain class of subjects.

"N" shows an exceptionally high figure before feeding, and "G" excep-

tionally low after feeding. The extraordinarily high results with "L" cannot be explained; the surgeon believed the specimen to be satisfactory. In the cases of "α" and "β" the numbers in brackets signify the number of hours after the single meal was taken. The fifth exps. with α and β were by a special method, and the indications were so slight as to be doubtful.

The increase in the aluminium content of the blood is in some degree a measure of the quantity of aluminium which has passed into the blood-stream.

Table II.

Subject	Sex and age	Aluminium found before feeding		
		1st exp.	2nd exp.	Adopted
α	M.	0 0 (3rd exp.)	0 0 (4th exp.)	0
β	M.	0 0 (3rd exp.)	0 0 (4th exp.)	— 0
A	M. 56	0·3	0	0·15
B	M.	0	—	0
E	—	0	—	0
G	M. 25	0	—	0
L	F.	0·1	—	0·1
M	F.	0	—	0
N	M. 25	0·2	0·3	0·25

Table III.

Subject	No. of daily meals	Aluminium found after feeding, parts per million			
		1st exp.	2nd exp.	3rd exp.	Adopted
α	1 (3)	0 0 (4th exp.)	0 0·1 (5th exp.)	0	0
	1 ($4\frac{1}{2}$)	0·3	0·4	0·2	0·3
	1 (48)	0·5	0·1	—	0·3
β	1 (3)	0 0 (4th exp.)	0 0·1 (5th exp.)	0	0
	1 ($4\frac{1}{2}$)	0·2	0·1	0·1	0·1
	1 (48)	0·3	0·7	—	0·5
A	1	1·2	1·1	1·3	1·2
B	2	0·4	0·2	0·2	0·3
E	5	0·2	0·2	0·5	0·3
G	7	0	0·1	0	0·03
L	12	5	4	—	4·5 (*v. infra*)
M	13	1·2	0·7	1·3	1·1
N	14	0·2	0·2	0·6	0·3

To what extent it is a measure is uncertain at present, because there are no data showing how quickly the aluminium is eliminated again. The observations with the subjects α and β signify that the passage is not very rapid, since it was not until 4 or 5 hours after feeding that the element was first discoverable, nor is the elimination very rapid, seeing that the aluminium lines were stronger at the end of 48 hours than after 4 or 5 hours. The results with subject A are in harmony with the foregoing; at the end of 24 hours the spectrograph revealed quite a substantial proportion, an increase of at least one part per

million. A notable feature of the results is that the quantity of aluminium after feeding is the same however many days the feeding has been continued.

Aluminium absorbed by the milk.

Experiments were made on two women, three cows and one goat. One of the cows and the goat were used twice, at times sufficiently far apart to allow of their being considered separate subjects, making in effect eight in all.

The order of experimenting was in general similar to that followed with blood. The aluminium was administered in combination with the food, as for the blood inquiry. In the case of cows turned out for the summer they were drenched with ½ oz. of alum.

All the results (Table IV) show clearly that aluminium passes fairly rapidly

Table IV. *Aluminium in milk.*

Subject	Before or after feeding	Dose, as alum	Interval (hours)	Al found; parts per million	Remarks
Cows:					
"Gay"	Before	—	—	(*a*) 0 (*b*) 0	After drenching with alum (in June)
,,	,,	—	—	(*c*) 0·3 (*d*) 0	
,,	After	(1½ ozs.)	9	(*a*) 0 (*b*) 0·4	,,
,,	,,	—	—	(*c*) 0·3 (*d*) 1·0	,,
"Brindle"	Before	—	—	(*a*) 0·3 (*b*) 0	,,
,,	,,	—	—	(*c*) 0 (*d*) 0·1	,,
,,	After	(1½ ozs.)	9	(*a*) 0·5 (*b*) 0·7	,,
,,	,,	—	—	(*c*) 0·3 (*d*) 0·1	,,
"Gay"	Before	—	—	(*a*) 0·2 (*b*) 0	—
,,	After	1½ ozs.	9	(*a*) 0·9 (*b*) 0·4	—
"Cowslip"	Before	—	—	(*a*) 0 (*b*) 0·1	—
,,	After	1½ ozs.	9	(*a*) 0·8 (*b*) 0·5	—
Goat A	Before	—	—	(*a*) 0 (*b*) 0	—
,,	After	½ oz.	5½	(*a*) 0 (*b*) 0	—
Goat B	Before	—	—	(*a*) 0 (*b*) 0	—
,,	After	½ oz.	14	(*a*) 0·3 (*b*) 0·2	—
Human A	Before (?)	—	—	(*a*) 0·1 (*b*) 0·1	As judged by the results these two samples have been transposed; possibly in the nursing home
,,	After (?)	28 grains	3½	(*a*) 0 (*b*) 0·02	
Human B	Before	—	—	(*a*) 0 (*b*) 0	
,,	After	28 grains	2	(*a*) 0·05 (*b*) 0·15	

into the milk. The procedure was to take the first sample of milk just before feeding with aluminised food, and the second sample from the next milking several hours later.

The intervals in the case of the human specimens were very short, and it is quite likely that this accounts for the negative results with subject A, especially with so small a dose, but it may be these two samples were transposed somehow, possibly in the nursing home.

It is interesting that the goat showed no absorption of aluminium the first time, and very little the second, although the dose was fairly large for the size of the animal.

The quantities found compare quite well among themselves if one omits two or three anomalous figures for the cows which were drenched, but with

these first specimens the work was largely of the nature of trial, and might quite fairly be omitted from the report, but they are included so that the whole of the information gleaned may be available.

There are various conditions to be observed in a closer study of the subject; for example, to discover the best interval between the time of feeding and the following milking, and whether the aluminium appears in the earlier or the later stages of the secretion. Also we have no knowledge as to how far the magnitude of the dose or its repetition affects the results. This is a matter of great physiological interest.

SUMMARY.

1. The chief feature of this inquiry is the application of the modern quartz spectrograph, in the absence of all reagents, to the estimation of aluminium in tissues.

2. It has been demonstrated that normal blood usually contains no aluminium, and when it does contain any, the proportion is very small.

3. A considerable quantity of aluminium is found in the blood of the same person after feeding with even one meal of aluminium-containing food.

4. Similar observations apply to the milk.

CCXXXIII. NOTE ON THE ACTIVATION OF OXIDO-REDUCTIONS BY CO-ZYMASE.

By HANS v. EULER and RAGNAR NILSSON.

From the Biochemical Institute of the University of Stockholm.

(*Received September 23rd, 1931.*)

The question of the existence of a co-reductase has frequently been discussed. The differences of opinion upon careful examination have proved often to originate from the fact that this problem has been understood in quite a different way by different authors. In a paper which recently appeared, Harden and Macfarlane [1931] have returned to this question and summarise their results as follows.

"The addition of lactate, succinate or methylglyoxal restored the power of reducing methylene blue to yeast preparations washed to a point at which no fermentation of hexosediphosphate occurred. From these results it is concluded that a co-enzyme is probably not required for the action of the reductase in yeast preparations."

We found previously [Euler, Nilsson and Runehjelm, 1927] in full agreement with Harden and Macfarlane, that the reduction of methylene blue, when performed in presence of lactate and succinate, takes place even in the absence of co-zymase.

On the other hand, co-zymase is required as an activator of the carbohydrate redoxase [Euler and Nilsson, 1926, 1, 2]. It was previously pointed out by Nilsson [1930], that a direct comparison between oxido-reductions in yeast and in muscle, in which different substrates take part as hydrogen donators is justified only in special cases. "Dazu kommt noch, dass die Specifität der bei verschiedenen Donatoren wirksamen Redoxasen nicht genügend erkannt wurde, so dass z. B. die Verbrennung von Milchsäure oder von Bernsteinsäure mit der Verbrennung von Kohlenhydratabbauprodukten in der Hefe und im Muskel direkt verglichen wurde, ohne dass man sich darüber klar machte, dass bei diesen verschiedenen Reaktionen vielleicht in sämmtlichen Fällen ganz verschiedene Enzyme oder Enzymsysteme wirksam sind."

Harden and Macfarlane state, that on the addition of co-zymase and hexosediphosphate to washed yeast fermentation occurs which may give rise to easily oxidisable reaction products, which in their turn cause a reduction of the methylene blue.

In such a case this reduction would be of a secondary character and it would consequently not correspond to the directly activated reaction in which the co-zymase acted as a co-reductase.

The possibility of explaining the chemical mechanism of the reduction of methylene blue in yeast in the manner recently suggested by Harden and Macfarlane has by no means been overlooked by us; it is noted in one of our earliest papers on this subject [Euler and Nilsson, 1926, 1].

However, our more recent results speak more and more in favour of the opinion that the reaction in which the co-zymase directly takes part is of an oxido-reductive character. As this view has already been discussed in a previous publication we only wish to emphasise here that reductions, which are activated by the co-zymase, take place even when fermentation or glycolysis is completely stopped by specific poisons. The product of the oxidation reaction which appears in this case has the composition of a monophosphoglyceric acid [Nilsson, 1930].

This conclusion is supported by the following facts.

Table 73 (p. 92) and Table 81 (p. 98) of Nilsson's paper show the reduction of acetaldehyde in presence and in absence of co-zymase after addition of NaF.

Table 74 (p. 93) shows that NaF completely inhibits all fermentation. From Tables 74 and 81 it will be seen that the reduction of acetaldehyde does not occur without co-zymase and that this reduction is not inhibited by NaF, when added in quantities which completely inhibit fermentation. A comparison with Table 72 (p. 92) shows that on the contrary the reduction of acetaldehyde is activated by such concentrations of NaF.

Since it became clear to us that the transformation of acetaldehyde in dried yeast consists in the reduction of the aldehyde, we used acetaldehyde instead of methylene blue in our oxido-reduction experiments, because it is possible to follow with this substance the course of the reduction from time to time.

We quote here, however, one of our earlier experiments with methylene blue, which shows that, in agreement with our reduction experiments with acetaldehyde, reduction of methylene blue by washed yeast + purified co-zymase and hexosediphosphate occurs in presence of fluoride.

Apozymase + phosphate + hexosediphosphate + methylene blue		Reduction time
+ Purified co-zymase	without NaF	1·5 minutes
	with NaF	1·75 „
Without co-zymase		More than 3 hours

The difference between the reduction times 1·5 and 1·75 lies within the limits of the errors of observation.

Concerning our opinion on the reactions which occur in this case and in the case of a normal fermentation we refer to the publication of Nilsson quoted above [Nilsson, 1930] and to a summarising paper by Euler and Nilsson [1930].

In this connection the question whether or not co-zymase acts as an activator of aldehydemutase is of special interest.

The fact that acetaldehyde, added to a suspension of dried yeast, disappears, was at first interpreted by us as a simple dismutation of the aldehyde and because co-zymase was found to be indispensable for the reaction the opinion was expressed that co-zymase has the character of a co-mutase [Euler and Myrbäck, 1927], which assumption was also in agreement with the result that co-zymase is identical with the co-enzyme of the reductase.

This question entered a new stage when it became apparent by the experiments of Nilsson that the acetaldehyde disappears in the presence of dried yeast, not by a simple dismutation but by a reduction very probably of the same type as the reduction of methylene blue.

This new fact caused a modification of our former theoretical views on this subject as is seen from Nilsson's paper [1930].

Thus the rôle of the co-zymase in oxido-reductions in the presence of yeast is cleared up to a certain degree; such is not the case with regard to the transformation of acetaldehyde in the liver, studied in detail by Parnas [1910]. By estimations of alcohol and acid Parnas has shown that it is here probably a question of a real aldehyde dismutation. But as all Parnas's experiments were carried out with mashed liver, or with raw liver juice, thus, with an enzymic material containing rather large amounts of carbohydrates, it is possible that a dismutation of mixed character also takes place, which may have influenced his results to some extent.

In this Institute the question has been again treated by Euler and Brunius [1928]. The most important results then obtained seem to be that purified and dialysed enzyme solutions, themselves inactive, were able to catalyse the aldehyde transformation after the addition of purified co-zymase.

Continuing this investigation we wished to compare the aldehyde transformation which is catalysed by the liver enzyme with the mixed dismutation as it takes place in suspensions of dried yeast.

These experiments also seem to support the opinion that in the liver a real acetaldehyde mutase occurs, which only acts in the presence of co-zymase. The fact that the transformation of acetaldehyde is activated by hexosediphosphate indicates, however, that an oxido-reduction may also occur analogous to that in fermentation by dried yeast, where hexosemonophosphate is proved to take part in a mixed dismutation with acetaldehyde [Nilsson, 1930].

Our results now obtained will be seen from the following tables.

1. *Action of phosphate in presence and in absence of hexosediphosphate.*

Enzyme solution: dialysed liver juice. Co-zymase: 140 Co/cc. ACo = 40,000.
Concentration of the acetaldehyde: 1·9 %.
Phosphate buffer: 0·67 M; $p_H = 6{\cdot}4$.
Concentration of the hexosediphosphate: solution of sodium candiolin, approximately 6 %.
Volume of the reaction mixture: 10 cc. In this volume 1 cc. acetaldehyde solution + 3 cc. enzyme solution + 2 cc. solution of co-zymase.

Acetaldehyde transformed in 2 cc. of the reacting mixture (mg.).

	No hexosediphosphate		1·5 cc. hexosediphosphate	
Mins.	No phosphate	2·5 cc. phosphate	No phosphate	2·5 cc. phosphate
30	0·89	0·83	0·83	1·42
60	1·44	1·48	1·61	2·27
90	1·94	2·17	2·08	2·76

2. *Action of co-zymase in presence and in absence of hexosediphosphate.*

All reagents as in the table above.

Volume of the reaction mixture: 10 cc., containing 1 cc. solution of acetaldehyde + 3 cc. solution of dialysed liver juice + 2·5 cc. phosphate buffer.

Acetaldehyde transformed in 2 cc. of the reacting mixture (mg.).

	No hexosediphosphate		1·5 cc. hexosediphosphate	
Mins.	No co-zymase	2 cc. co-zymase	No co-zymase	2 cc. co-zymase
30	0·10	0·85	0·25	1·40
60	0·12	1·55	0·39	2·25
90	0·23	2·21	0·76	3·03

Summary.

1. For aldehyde transformation, when catalysed by dialysed liver juice and purified co-zymase, phosphate is not required. There is thus a difference from the mixed dismutation, as it occurs in solutions fermented by dried yeast.

2. The transformation of acetaldehyde in dialysed liver juice is increased by the addition of hexosediphosphate or of phosphate.

3. Both in the presence and in the absence of hexosediphosphate co-zymase is indispensable for the transformation of acetaldehyde by liver juice.

We are indebted to Mr Bengt Andersson for valuable help in the experiments recently repeated.

References.

Euler and Brunius (1928). *Z. physiol. Chem.* **175**, 52.
—— and Myrbäck (1927). *Z. physiol. Chem.* **165**, 28.
—— and Nilsson (1926, 1). *Z. physiol. Chem.* **162**, 72.
—— —— (1926, 2). *Z. physiol. Chem.* **162**, 264.
—— —— (1930). *Skand. Arch. Physiol.* **59**, 201.
—— Nilsson and Runehjelm (1927). *Z. physiol. Chem.* **169**, 123.
Harden and Macfarlane (1931). *Biochem. J.* **25**, 818.
Nilsson (1930). *Svenska Vet. Akad. Arkiv Kemi* 10 A, nr. 7.
Parnas (1910). *Biochem. Z.* **28**, 274.

CCXXXIV. "RESTING" BACTERIA.

By BRIAN RATCLIFFE SANDIFORD
and WALTER REGINALD WOOLDRIDGE.

From the Divisions of Bacteriology and of Biochemistry, the London School of Hygiene and Tropical Medicine.

(*Received October 13th, 1931.*)

During recent years much work has been reported upon the existence and properties of various bacterial oxidising enzymes. For most of these researches thick suspensions of well-washed bacterial cells in physiological saline or Ringer's solution have been used. These bacterial preparations, when used under conditions such that growth cannot occur [Quastel and Whetham, 1924], have been called "resting" bacteria, a term suggested by analogy with that of "resting" muscle, a similar preparation of well-washed muscle tissue. Although the term "resting" bacteria appears to be justified in the biochemical sense it has been a somewhat unfortunate choice for the bacteriologist if it be taken to imply that "resting" bacteria are normal viable cells which are only unable to proliferate because they are used under conditions unsuitable for their growth; *e.g.* in the absence of necessary food materials or at unfavourable temperatures. It has seemed to him unjustifiable to describe bacterial suspensions as "resting" when no effort was made in the early work to demonstrate any connection between the different stages of the experiments and the total and viable counts of the organisms present. At that time the main concern was to simplify the complexity of bacterial processes as much as possible so that certain of their systems could be more rigorously examined, and hence the actual relationship between the enzymic mechanisms and the life or death of the cells present was not emphasised. In 1927 Quastel and Wooldridge [1927, 1, 2] showed that bacteria previously submitted to various treatments, *e.g.* toluene, chloroform, permanganate *etc.*, so that they would no longer proliferate when inoculated on to agar slopes, nevertheless exhibited little variation in the enzymic activity of many of the mechanisms studied. In these experiments no actual counts were made. After discussing at some length the question of whether the activations of the cell are due to the latter acting as an organised whole, the authors conclude that "we have indeed no evidence to support a contention that the reactions of the organism are dependent upon its survival as a living unit...." Later Cook and Stephenson 1928], who were examining the behaviour of bacterial suspensions in Ringer's solution in a Barcroft differential manometer, *i.e.* in the presence of oxygen,

carried out a series of bacterial counts on their preparations both before and after treatment with ultra-violet light and compared these counts with the oxygen uptake. They conclude "that the oxidation phenomena studied are the work of dead equally with living organisms."

The bulk of the work with "resting" bacteria has been effected under anaerobic conditions, usually at 45°, and it was thought advisable to make total and viable counts of organisms used under these conditions, not only at the beginning and end of the experiments, but at various intervals over a period of several hours. The importance of these Thunberg tube-methylene blue experiments to the bacteriologist was emphasised as early as 1925 [Quastel and Wooldridge, 1925; Quastel and Stephenson, 1925] by the correlation of the results obtained from a study of the enzymic powers of various organisms with their ability to grow upon synthetic media. Since then the term "resting" bacteria has invaded bacteriological literature to such an extent that it has become more than ever necessary to demonstrate the true nature of these suspensions from the angle of the strict bacteriologist. Accordingly the work described in this paper was undertaken.

Experimental.

An organism is said to activate a substrate, examined by the anaerobic methylene blue technique, when the methylene blue is decolorised within a period of 2 hrs., whilst a control experiment with the organism in the absence of the substrate does not reduce the methylene blue at all. Frequently the reduction time with an activated substrate is only a few minutes, but as occasionally experiments have been carried out, and the results reported, over a period of 22 hrs. it was decided to examine the organism over this long period. Accordingly series of Thunberg tube experiments have been put up with *B. coli* suspensions in the presence of various substrates and these have been incubated anaerobically at 45° for different periods of time ranging up to 22 hrs. At the end of the incubation periods total and viable counts of the organisms present in the suspensions have been made. In parallel with these experiments other series of tubes have been incubated so that the effect of the incubation period upon the enzymic activity of the organism could be ascertained. In this way it has been possible to obtain curves showing the variation of total and viable counts with the period of incubation and also to compare these variations with any changes discovered in the enzymic activity.

In the preparation of the organism an 18-hr. broth culture of *B. coli* was inoculated into Roux bottles containing caseinogen digest broth. After 36 hrs. incubation at 37° the organism was centrifuged free of broth, washed twice by centrifugation with sterile saline, made up to a suitable suspension with sterile saline, aerated for half-an-hour, and the organism then stored at 0° until required for use. All experiments were made with an organism that had not been prepared for more than 5 days. For actual use

the suspension was adjusted, by comparison with turbidity controls of *B. coli*, so that its final dilution in the Thunberg tube would contain between 500 and 1000 million cells per cc.

For the Thunberg tube experiments each tube contained 2 cc. of sterile phosphate buffer, p_H 7·4, 1 cc. of sterile substrate, $M/20$ glucose, lactate, formate or succinate—all adjusted to p_H 7·2 and 1 cc. of the *B. coli* suspension. Some of the tubes were incubated with 1 cc. of methylene blue (1/5000 solution in water) whilst to the others 1 cc. of sterile physiological saline was added instead. The organism was added last, the tube closed, immediately evacuated, the side-arm filled with sterile water and placed in the water-bath at 45°. The first of each series of experiments was only left in the bath for 30 secs. when it was removed, cooled under running water, the water removed from the side-arm and the tube carefully opened so that no grease was allowed to descend into the fluid. Total (*i.e.* living and dead cells) and viable counts were begun on this suspension almost immediately. Viable counts were made similarly after incubation for the following times: 20 mins., 40 mins., 1 hr., 2 hrs., 3 hrs., 4 hrs., 5 hrs., 6 hrs. and 22 hrs. Total counts were estimated after 1-hr., 6-hr. and 22-hr. incubation periods. At times various tubes of a series were examined for purity by inoculating nutrient media and incubating both at 37° and at 45° and sometimes at 53° in order to ascertain whether any thermophilic or other contaminants were present. In all these experiments the suspensions were found to consist solely of *B. coli*.

For assessing the total number of organisms present the technique followed was that described by Wilson [1922]. In this the Helber chamber is used and the unstained organisms are counted against a dark background.

The viable counts were made by the agar roll-tube method discussed in the same paper.

All dilutions were effected by the use of dropping pipettes [Donald, 1915]. The margin of error of the results given in the present paper may be slightly greater than usual owing to the fact that at times the dropping pipettes became contaminated with grease, which would affect somewhat the size of the drops.

The experiments for examining the effect of the anaerobic incubation at 45° upon the activity of the enzymes were carried out in Thunberg tubes just as for the bacterial counts. At the end of the required incubation the tube was cooled, the water in the side-arm withdrawn and the stopper carefully opened. The stopper and neck of the tube were cleaned with a dry rag and the former re-greased. If the incubation had taken place in the absence of methylene blue this was now added, the tube closed and re-evacuated and the time required for the reduction of the dye on replacing in the bath at 45° noted. If the first incubation had taken place in the presence of methylene blue, then before the second evacuation the contents of the tube were well shaken to oxidise the dye, and just before immersion in the bath the proportion of oxidised dye present was ascertained by comparison with a series

of controls. Any necessary correction was then made in the second reduction time before comparing this with that previously obtained.

Results representative of the many obtained are set forth in the following tables or shown graphically in the figures.

Discussion of results.

Viable counts. The curves of Fig. 1 show that the number of viable organisms present in bacterial suspensions incubated anaerobically at 45° in the presence of various substrates, but in the absence of methylene blue, decreases with increase in the period of incubation.

If, in addition to the substrate, methylene blue be added, there is a fairly rapid diminution in the number of viable organisms within the first hour, but this is followed by an increase in numbers in all instances except with

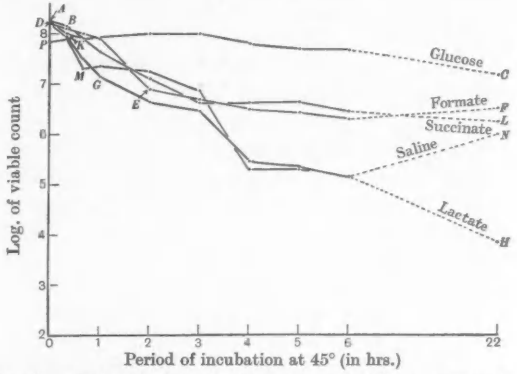


Fig. 1. Showing the relationship between the number of viable organisms and the previous period of incubation at 45° in the presence of the named substrate but in the absence of methylene blue. Glucose = curve *ABC*. Formate = curve *DEF*. Lactate = curve *DGH*. Succinate = curve *AKL*. Saline = curve *PMN*. N.B. The time-scale is greatly reduced between 6 hrs. and 22 hrs.

the saline series (see Fig. 2). This increase tends to balance the previous decrease but subsequently the viable count diminishes again. With the saline-methylene blue series no growth at all was obtained from the dilutions planted from the 2-hr. to 6-hr. tubes, but this was probably the result of diluting the suspensions too much, for with the 22-hr. tube, where the suspension was inoculated undiluted, some growth, although less thick than that obtained in the earlier tubes, was noted. It is therefore evident that the increase in the number of viable organisms observed with the other series is absent from that with saline and methylene blue.

It may be possible to explain these results from a consideration of the number of viable organisms involved. There is evidence to suggest [see Bail, 1929] that many organisms will only grow upon media, provided that the

initial concentration of viable organisms is below a certain critical value. This fact might explain the results obtained with the lactate and formate series, for if the initial concentration of viable organisms was already at the critical value in the experiments without the dye then no growth would be expected but in the methylene blue tubes the initial viable count is much less, possibly below this critical "saturation" value, and growth might take place. It is more difficult to explain the glucose results in this way as with these the initial concentrations of viable organisms differ little.

An alternative biochemical explanation may be suggested, depending upon the presence of methylene blue in those tubes where an increase in number of viable organisms is noticed. Methylene blue, in addition to its known inhibitory effect on many enzymes, will also act as a hydrogen acceptor and so will facilitate the oxidation of such hydrogen donators as glucose, lactate and formate in the presence of the organism. These oxidation changes may

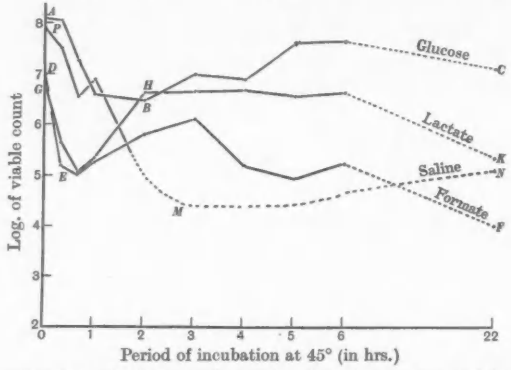


Fig. 2. Showing the relationship between the number of viable organisms and the previous period of incubation at 45°, in the presence of a substrate and of methylene blue. Glucose = curve *ABC*. Formate = curve *DEF*. Lactate = curve *GHK*. Saline = curve *PMN*. N.B. The time-scale is greatly reduced between 6 hrs. and 22 hrs.

result in the formation of some substance essential to or definitely stimulatory of growth and would, in addition, yield a supply of energy and, as a result of one or both of these factors, growth might ensue. This growth would be facilitated by the presence of some nitrogenous materials probably made available from the dead cells. Growth will only occur when in addition to the hydrogen acceptor, methylene blue, a hydrogen donator is present and hence no increase in the viable count would be expected in the saline-methylene blue series of experiments—an expectation borne out by experiment. But even in the presence of a hydrogen donator growth will not continue for long under the conditions of these experiments for two reasons; firstly the methylene blue will soon be entirely reduced to the leuco-compound and, although this might be re-oxidised by protein products, the action would continue for but a short time; secondly the inhibitory action of the dye would become increasingly

important. Hence after a short period of growth the number of viable organisms would again decrease. This early cessation of growth might, of course, be due to other factors, such as the lack of adequate nitrogenous material. The above explanation, however, is tentative and needs further work for corroboration. A further fact that should be noticed is that the delay in growth corresponds to the ordinary lag phase of bacterial growth on nutrient media and so the changes noticed may be due to the same causes as are responsible for this lag phase. What relation the phenomena described in the above explanation of the results obtained may have to the lag phase, if any, still remains to be determined.

An examination of the figures given in Table I shows that the total number of cells remains practically stationary throughout the period of incubation. At the beginning the ratio of viable organisms to total cells varies between 1 : 2 and 1 : 7 in the different experiments, except with the lactate-methylene blue series, but at the end of the period of incubation this ratio is always greatly altered—varying between 1 : 200 and 1 : 6,000,000. It follows therefore that if the enzymic activity of these suspensions were examined in parallel with the growth series, then the enzymic activity should either be little changed, if it is independent of whether the cell is alive or dead, or it should fall off greatly and become immeasurable by our technique if the activity is due solely to the presence of living organisms. This holds only if the velocity of the enzyme reaction as measured by the Thunberg technique is proportional to the concentration of the enzyme present. That this is so is shown by the curves given in Fig. 3. The results given in Table II show that for practical purposes the enzymic activity remains constant, the greatest change recorded being a sixfold increase in the reduction time. These results therefore conclusively demonstrate that the enzymic activity is not solely dependent upon the living cell but appears to be a property both of cells that are unable to grow, even given suitable conditions, and of cells that are still alive and only unable to grow during the experiment because of the conditions of this experiment. The implication that preparations described as "resting" bacteria consist mainly of cells which are in a state of suspended animation as regards growth is incorrect, although the claim that the synthetic reactions associated with growth are largely inhibited by their use under specified conditions remains valid. The term "resting" bacteria therefore should signify a suspension of washed bacterial cells, some alive and some dead, used under conditions such that proliferation, that is increase in the number of living cells, is inhibited during the usual period of observation. Then the reactions studied will generally be those associated with both the living and the dead cells.

As the cardinal point in this definition is that multiplication of the bacteria is inhibited during the use of "resting" bacteria it is obviously essential, if the term be applied at all generally to the use of suspensions of washed bacterial cells, that control experiments on bacterial counts be conducted in parallel with the biochemical reactions being studied. Under conditions such

Table I. *Showing the effect of varying the substrate upon the total and viable counts of organisms present in suspensions of* B. coli *incubated anaerobically at 45°. Except for the series marked with an asterisk, the results given (no. per cc.) with one substrate were obtained with the same bacterial suspension.*

	Substrate	Count	0 min.	20 mins.	40 mins.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	22 hrs.
1.	Saline	Total	363,000,000	—	—	323,000,000	—	—	—	—	327,000,000	330,000,000
2.	,, +M.B.	Total	333,000,000	—	—	330,000,000	—	—	—	—	357,000,000	367,000,000
3.	,, +M.B.	Viable	73,720,000	30,510,000	3,390,000	8,052,000	0	0	0	0	0	133,900
4.	,, *	Viable	66,900,000	83,030,000	42,450,000	23,030,000	16,940,000	7,340,000	191,220	205,700	125,700	983,700
5.	Lactate	Total	1,000,000,000	—	—	1,220,000,000	—	—	—	—	1,212,000,000	894,000,000
6.	,,	Viable	171,000,000	123,700,000	62,700,000	44,910,000	11,870,000	5,932,000	308,600	343,000	291,000	25,710
7.	,, +M.B.	Total	1,018,000,000	—	—	1,034,000,000	—	—	—	—	876,000,000	1,040,000,000
8.	,, +M.B.	Viable	5,453,000	471,500	120,000	222,900	4,286,000	4,629,000	4,887,000	3,517,000	4,286,000	214,500
9.	,, *	Viable	210,000,000	90,000,000	33,000,000	14,600,000	4,661,000	3,000,000	271,500	225,000	132,000	7,310
10.	Formate	Total	616,000,000	—	—	654,000,000	—	—	—	—	570,000,000	566,000,000
11.	,,	Viable	194,800,000	46,610,000	67,790,000	10,590,000	9,745,000	4,661,000	34,300	154,300	56,010	8,570
12.	,, +M.B.	Total	694,000,000	—	—	724,000,000	—	—	—	—	674,000,000	450,000,000
13.	,, +M.B.	Viable	9,322,000	1,611,000	124,300	185,700	638,300	1,277,000	165,000	85,270	178,400	9,840
14.	,, *	Viable	152,400,000	119,900,000	93,240,000	74,730,000	8,664,000	5,484,000	3,368,000	2,887,000	1,987,000	3,273,000
15.	Glucose	Total	832,000,000	—	—	1,002,000,000	—	—	—	—	762,000,000	858,000,000
16.	,,	Viable	169,400,000	135,500,000	77,900,000	266,400,000[1]	103,400,000	96,580,000	64,390,000	50,860,000	45,750,000	13,560,000
17.	,, +M.B.	Total	830,000,000	—	—	878,000,000	—	—	—	—	747,000,000	628,000,000
18.	,, +M.B.	Viable	118,600,000	110,100,000	18,880,000	4,237,000	2,966,000	10,590,000	7,628,000	38,140,000	43,220,000	118,870,000
19.	,, *	Viable	13,920,000	10,490,000	10,670,000	10,250,000	7,509,000	7,750,000	6,991,000	2,030,000	140,700	—
20.	Succinate	Viable	165,900,000	111,100,000	84,330,000	43,190,000	12,850,000	4,230,000	4,268,000	3,210,000	2,762,000	1,663,000

[1] The suspension in this tube was badly affected with grease.

as those used by Quastel and Wooldridge [1927, 1, 2; 1928] in which the organisms are initially exposed to high concentrations of inhibitors such as toluene, $KMnO_4$ *etc.*, this precaution is not so necessary. But the fact that growth has been observed to take place under conditions thought to be inhibitory of any increase in numbers, as described in the present paper, makes such controls generally advisable. It is true that this increase in numbers

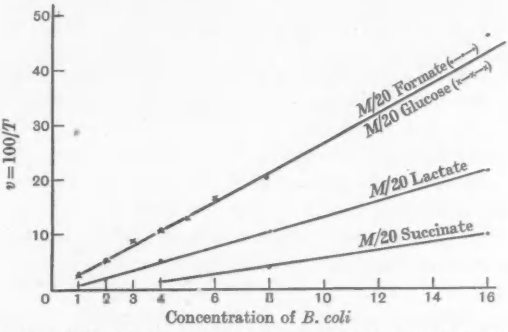


Fig. 3. Showing the relationship between the concentration of "resting" *B. coli* and the velocity of reduction of methylene blue ($v = 100/T$, where T = time of reduction in mins.) in the presence of various substrates. Concentration 16 of *B. coli* = approx. 5×10^8 cells per cc.

Table II. *Showing the effect of an initial anaerobic incubation of variable duration at 45° and* p_H *7·4 upon the times (in mins.) taken by "resting"* B. coli *to reduce* M/*25,000 methylene blue solution, the preliminary incubation taking place in the presence or the absence of the dye, as noted at the head of each column.*

Period of first incubation at 45°	Lactate	Lactate +M.B.	Formate	Formate +M.B.	Glucose	Glucose +M.B.	Succinate	Succinate +M.B.
0 min.	10	8	8	9	13	12	8	8
5 mins.	10	12	12	17	15	15	6	8·5
10 ,,	8·5	9	7	7	13	14·3	7	8
15 ,,	12	7	7	8	11	19	8	8
20 ,,	9	11	8	8·5	11	21	10	8
30 ,,	12	10	11	7	14	13	10	11
45 ,,	13	10	8	6·5	11·3	12	14	12
1 hr.	11	13	6·5	8	11	12	14	15
2 hrs.	8	14	7	9	10	14·3	20	21
3 ,,	14	10·5	6·5	8	12·5	11·3	18	20
4 ,,	11	16	6·5	11	12·5	10·7	18	22
5 ,,	10	16	7	6·5	12	9·5	14	20
6 ,,	11	18	7	8	10	12·7	19	21
22 ,,	20	49	9	18	12·3	17·7	51	70

was small and transient and that it only occurred after the usual period of observation in these enzyme studies. (In the great majority of dehydrogenase experiments reported in the previous work upon these bacterial enzymes the period of observation did not exceed 30 mins. and so the conclusions of, and deductions drawn from, this work are in no way invalidated.) Nevertheless, owing to the recent extension of the use of so-called "resting" bacteria,

especially in America, it seems advisable to discontinue the use of this term before absolute confusion results. The only justification for the use of the term is if bacterial growth definitely does not take place, and as this is generally doubtful and rarely proven it is preferable that future workers along these lines should simply allude to the use of washed bacterial cells and describe the conditions under which they are employed.

Summary.

1. It is shown that preparations described as "resting" bacteria consist both of cells that will proliferate when inoculated upon suitable media and of cells that are unable to grow under such conditions.

2. The total number of cells remains practically constant throughout long periods of anaerobic incubation at 45° in the presence of various substrates with or without methylene blue.

3. In the absence of the dye the number of viable organisms steadily decreases, whereas in the presence of methylene blue the viable count first shows a fall followed by a temporary rise, subsequently falling more slowly. An explanation of this phenomenon is suggested.

4. The enzymic activity of these preparations towards the various substrates used is shown to remain practically constant throughout the period of incubation.

5. The enzymic activities examined in this work are not solely dependent upon the presence of living cells; both viable and dead cells are active.

6. As the number of living cells may sometimes increase under conditions thought to be inhibitory of growth it is essential that control experiments assessing the number of viable organisms be conducted whenever the term "resting" bacteria is used.

7. It is preferable to discontinue the use of this term entirely and to refer simply to washed bacterial cells used under certain specified conditions.

REFERENCES.

Bail (1929). *Z. Immunität.* **60**, 1.
Cook and Stephenson (1928). *Biochem. J.* **22**, 1368.
Donald (1915). *Lancet*, ii, 1243.
Quastel and Stephenson (1925). *Biochem. J.* **19**, 660.
—— and Whetham (1924). *Biochem. J.* **18**, 519.
—— and Wooldridge (1925). *Biochem. J.* **19**, 652.
—— —— (1927, 1). *Biochem. J.* **21**, 148.
—— —— (1927, 2). *Biochem. J.* **21**, 1224.
—— —— (1928). *Biochem. J.* **22**, 689.
Wilson (1922). *J. Bact.* **7**, 405.

CCXXXV. THE NITROGEN METABOLISM OF THE LUPIN SEEDLING.

By PHYLLIS McKIE.

From the Department of Biochemistry, Westfield College, London.

(*Received October 21st, 1931.*)

The following investigation was undertaken for two reasons; to follow the nitrogen metabolism of the young plant through successive stages of growth and thus to arrive at some estimate of the part played by different nitrogenous metabolites, and to provide a preliminary survey for the much more complicated problem—at what stage and by what means is the pyrrole ring synthesised in the plant?

The first stages of germination of a seed containing reserve protein involve the breakdown of that protein. It becomes a matter of considerable interest therefore to examine the various products of this breakdown and to gain some knowledge of the nitrogen compounds which are produced by the plant from its reserves, prior to their use in synthesis. It is apparent, as will be shown, that protein synthesis begins after about the 18th day of germination, when the seed reserves have been (for the lupin) largely depleted. The main seat of metabolic activity of such synthesis is probably in the leaves. In a young seedling, in which the leaves form a small proportion of the bulk of the plant, it is misleading to confine analysis solely to these parts, and up to the 82nd day of growth the whole seedling has been taken for analysis. The material from each sowing (about 12 plants) was mixed, discarding any obvious abnormalities. In this way minor differences, inseparable from the growth of individual plants, were eliminated. In the lupin, asparagine represents about one-half of the protein decomposition products in the seed, and the nitrogen metabolism of the lupin seedling is therefore peculiar in this respect, but asparagine is probably only one of several labile intermediate substances of protein breakdown, and the subsequent course of protein re-synthesis may give some qualitative idea of this process in other seedlings.

Experimental.

Lupin seeds (*Lupin luteus*), were grown in large pots containing a granular soil and sand mixture in a cool greenhouse throughout the summer and early autumn of 1930. Twelve pots, each producing ultimately about a dozen plants, were used for each sowing. Regulation of the temperature of the greenhouse ensured approximately the same conditions throughout the experiment. In

one lot only were conditions adverse; Lot 1, a 31 day period lasting from October 25 to November 29, which coincided with an exceptionally cold spell of weather. Artificial heat above the average cool temperature was not favourable, and growth was stunted accordingly.

Preparation and analysis of material.

The plants were released from the soil by turning out the pot and gently disengaging the root and root-hairs as much as possible without breaking. Ten minutes' immersion in water and very gentle cleaning with the fingers were sufficient to free the seedlings entirely from soil, They were quickly air-dried on sheets of blotting-paper, and measured for total length, number of leaves and lateral roots.

After 1 minute's immersion in boiling water to arrest growth and enzyme action, the seedlings were dried as completely as possible on blotting-paper and transferred to spirit. This enables analyses to be undertaken at leisure, and avoids the disadvantage of high temperature drying of the partially wet material; it also affords a means of isolating the alcohol-insoluble asparagine which appears in quantity after a few days' growth. A deposit of asparagine appears at the bottom of the jar, and in the subsequent stages—rapid air-drying at 50°—the rest of the asparagine crystallises out on the dry material, and can readily be detached. The asparagine-free material is ground to powder in an end-runner mill and dried at 100° to constant weight. Analyses for total nitrogen (Kjeldahl) only were carried out on the dry material.

Extraction of material. The dried and weighed material was intimately ground in an end-runner mill with 100 cc. of ammonia-free water for 20 minutes and filtered through fine muslin. The process was repeated twice more, the last residue being pressed as dry as possible.

Residue. This was dried at 100° to constant weight and analysed for total nitrogen.

Filtrate. The combined filtrates were made up to 500 cc. with ammonia-free water, made antiseptic with a little toluene and analysed.

Total nitrogen. Of the dry material weights averaging 0·02 g., or of the extract 10 cc. evaporated to dryness in the Kjeldahl flask, were taken. The modified Kjeldahl method used by Robinson [1929], was found satisfactory. A clear liquid was always obtained after 20 minutes' boiling, and boiling was continued for a further 10 minutes. Distillations were carried out in the usual apparatus with the addition of 30 cc. of ammonia-free water, 5 cc. of 25 % sodium sulphide, 5 cc. of *N* copper sulphate, and 25 cc. of alcohol, aerated by acid-washed air during the 20 minutes' boiling, the ammonia being received in $N/10$ H_2SO_4. Controls had established the accuracy of this procedure.

Protein. 10 cc. of extract were diluted to 100 cc. with absolute alcohol, added slowly with constant shaking. After standing for 36 hours the precipitated protein was collected in a Jena glass crucible and dried in a desiccator to constant weight. Nitrogen was calculated in this protein as 12·8 %.

Proteose. The method of Wasteneys and Borsook [1917] was found most suitable. 20 cc. of extract were employed.

Nitrate. This was determined on 50 cc. of extract by reduction with Devarda's alloy as recommended by Chibnall [1922].

Amino-acids. The Sørensen method of estimation of amino-acid content was finally adopted, 10 cc. being used for each titration.

Ammonia. Free ammonia, which is usually small in amount, is best estimated by the Folin aeration method, using solid anhydrous sodium carbonate. 30 cc. of extract *plus* 2 g. of anhydrous sodium carbonate were aerated in the Folin apparatus for 1 hour at 15°, the ammonia being received in 25 cc. of $N/100$ H_2SO_4.

Amides. The amide content was taken as represented by that amount of ammonia *minus* the free ammonia, which is yielded by the Folin method after 5 hours' boiling with 4 % HCl, by the method of Sachsse [1873]. 5 cc. of extract and 20 cc. of acid were taken for each estimation.

Asparagine. A considerable proportion of the asparagine, when present, crystallises out from the material in spirit, and, after the removal of the seedlings, can be filtered off through a Jena glass crucible, dried and weighed. The remainder crystallises on the surface of the seedlings during the air-drying and can be detached with a small brush, collected and weighed.

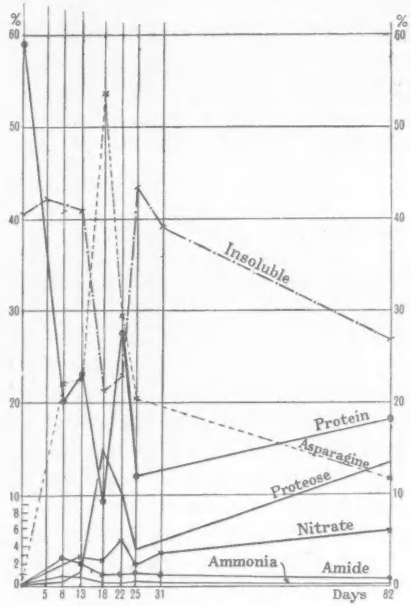


Fig. 1.

Examination of the curves, Fig. 1, shows that over a period of 81 days' growth from the seed, protein, insoluble nitrogen compounds, and asparagine represent by far the greater part of the total nitrogen.

In the seed, soluble protein and nitrogenous material insoluble after the three extractions with water account for the whole of the nitrogen.

After 5 days' growth, soluble protein has decreased sharply, and this is the more marked at 8 days, when there has been a fall of protein nitrogen from 59·2 % (in the seed) to 20·2 %. After a slight fluctuation, the level falls still further, until at the 18th day it is only 9·8 %, the minimum value (Table I).

Table I. *Variation in protein content.*

	Age (days)	Protein dry weight g.	Protein as % of total solid	Protein-nitrogen as % of total solid	Protein-nitrogen as % of total nitrogen
X.	0	13·4	29·6	3·8	59·2
B.	5	—	—	—	—
A.	8	0·395	12·95	1·65	20·02
F.	13	0·420	16·21	2·07	23·4
C.	18	0·410	14·68	1·88	9·81
G.	22	0·740	35·34	4·52	27·8
E.	25	0·320	13·69	1·75	12·01
D.	31	0·735	18·5	2·24	15·03
H.	82	0·665	22·37	2·34	18·5

(N.B. Protein nitrogen is calculated as 12·8 %.)

Following closely this decrease in protein there is a sharp decrease in insoluble nitrogenous material, which reaches its minimum on the 22nd day at 14·9 % (original seed value = 41·09 %) (Table II).

Table II. *Variation in content of insoluble nitrogen compounds.*

Age (days)	Nitrogen as % of dry residue	Residue-nitrogen as % of total dry weight	Residue-nitrogen as % of total nitrogen
0	8·8	2·64	41·09
5	—	4·94	43·96
8	8·8	3·98	61·3
13	7·07	3·61	41·5
18	6·7	3·97	20·7
22	5·3	2·42	14·9
25	8·54	6·34	43·55
31	8·22	5·8	38·9
82	5·29	3·33	26·3

Concomitantly with this fall asparagine increases rapidly during the early days of growth. It is absent from the ungerminated seed, but after 18 days' growth the asparagine represents 53·7 % of the total nitrogen. This is its maximum value, and during subsequent growth it diminishes rapidly in the next 12 days, corresponding to a synthesis of protein, and thereafter diminishes slowly. Asparagine appears, therefore, to represent the chief metabolic product in the first stages of growth and to represent, with the protein, the greater part of the nitrogenous material of the seedling (Table III).

Table III. *Variation in asparagine content.*

	Age (days)	Total solid dry wt. g.	Asparagine g.	Asparagine-nitrogen g.	Asparagine-N as % of dry wt.	Asparagine-N as % of total N
X.	0	—	0	0	0	0
B.	5	—	—	—	—	—
A.	8	3·0487	0·2521	0·0535	1·75	21·23
F.	13	2·5924	0·2377	0·0503	1·94	22·3
C.	18	2·7928	1·3292	0·2818	10·29	53·7
G.	22	2·0945	0·4724	0·1002	4·78	29·4
E.	25	2·3367	0·3146	0·0667	2·99	20·5
D.	31	3·9735	0·4087	0·0867	2·18	14·6
H.	82	2·9729	0·189	0·04	1·35	10·6

Proteoses are seen to be an immediate decomposition product of the protein and insoluble nitrogenous material, the curve for proteose nitrogen rising from zero in the seed to a maximum at 14·2 % after 18 days' growth. While protein is being rapidly synthesised during the next 7 days, proteoses diminish, but in the subsequent slower growth of the young plant, proteoses increase gradually with a corresponding increase of protein, keeping as would be expected, at a lower level throughout (Table IV).

Table IV. *Variation in proteose content.*

Age (days)	Total nitrogen of extract g.	Nitrogen other than protein or proteose g.	Nitrogen of protein *plus* proteose g.	Nitrogen of protein g.	Nitrogen of proteose g.	Proteose-nitrogen as % of total solid	Proteose-nitrogen as % of total nitrogen
5	0·198	0·127	0·071	0·050	0·021	0·07	0·85
8	0·175	0·084	0·091	0·053	0·038	0·15	1·72
13	0·253	0·125	0·128	0·052	0·076	2·72	14·2
18	0·241	0·112	0·129	0·095	0·034	1·68	10·3
22	0·273	0·220	0·059	0·046	0·013	0·55	3·78
25	—	—	—	—	—	—	—
31	0·335	0·218	0·117	0·069	0·048	1·66	13·13
82	—	—	—	—	—	—	—

The behaviour of the labile metabolic products shows an interesting parallel. Always low and never representing in total more than 9 % of the total nitrogen, and averaging ammonia and amino-acids, 0·2 %, amides and nitrates 3 %, they appear in the seedling after 5 days' growth, and are at their maximum during the period of rapid change, 8 to 18 days, corresponding to the very rapid fall of protein and the equally rapid rise of asparagine. With the exception of nitrates they maintain a fairly constant low level throughout the subsequent slower periods of growth. Nitrates tend to increase slightly as the seedling acquires more leaves and leaf metabolism thereby increases (Tables V, VI, VII and VIII).

The apparently insignificant part played in the seedling by amides, amino-acids, nitrates and ammonia, as suggested by the curves, is probably however misleading, for at no time is it tc be expected that these substances, as much the elements of synthesis as of degradation, would accumulate. Thus, while the breakdown of protein to amino-acids, amides and ammonia may be rapid

Table V. *Variation in amide content.*

	Age (days)	Total solid dry wt. g.	N as amide *plus* ammonia g.	N as ammonia g.	N as amide g.	Amide-N as % of total solid	Amide-N as % of total nitrogen
X.	0	—	0	0	0	0	0
A.	8	3·0487	0·0105	0·0026	0·0079	0·259	3·15
F.	13	2·5924	0·0084	0·0021	0·0063	0·243	2·76
C.	18	2·7928	0·0091	0·0014	0·0077	0·276	1·41
G.	22	2·0945	0·0063	0·0014	0·0049	0·234	1·44
E.	25	2·3367	0·007	0·0013	0·0057	0·244	1·65
D.	31	3·9735	0·0091	0·0012	0·0079	0·199	1·34
H.	82	2·9729	0·0056	0·0014	0·0042	0·141	1·11

Table VI. *Variation in ammonia content.*

	Age (days)	Total solid dry wt. g.	Ammonia g.	Ammonia nitrogen g.	Ammonia-N as % of total solid	Ammonia-N as % of total nitrogen
X.	0	—	0	0	0	0
A.	8	3·0487	—	0·0026	0·084	1·02
B.	5	2·3628	—	0·00059	0·0257	0·26
F.	13	2·5924	—	0·0021	0·081	0·93
C.	18	2·7928	—	0·0014	0·050	0·22
G.	22	2·0945	—	0·0014	0·067	0·41
E.	25	2·3367	—	0·0013	0·055	0·38
D.	31	3·9735	—	0·0011	0·029	0·19
H.	82	2·9729	—	0·0014	0·047	0·73

Table VII. *Variation in nitrate content.*

	Age (days)	Total solid dry wt. g.	Nitrate-nitrogen g.	Nitrate-nitrogen as % of dry wt.	Nitrate-nitrogen as % of total nitrogen
X.	0	—	0	0	0
B.	5	—	—	—	—
A.	8	—	—	—	—
F.	13	2·5924	0·0071	0·2746	3·15
C.	18	2·7928	0·0156	0·56	2·92
G.	22	2·0945	0·0171	0·8155	5·01
E.	25	2·3367	0·0071	0·3044	2·09
D.	31	3·9735	0·0199	0·5013	3·36
H.	82	2·9729	0·0227	0·7656	6·01

Table VIII. *Variation in amino content.*

	Age (days)	Total solid dry wt. g.	Amino-nitrogen g.	Amino-nitrogen as % of dry wt.	Amino-nitrogen as % of total nitrogen
X.	0	0	0	0	0
B.	5	—	—	—	—
A.	8	3·0487	0·0023	0·076	0·92
F.	13	2·5924	0·0022	0·083	0·954
C.	18	2·7928	0·0016	0·056	0·29
G.	22	2·0945	0·0008	0·040	0·24
E.	25	2·3367	0·0010	0·044	0·30
D.	31	3·9735	0·00103	0·026	0·17
H.	82	2·9729	0·0005	0·016	0·13

and extensive, the removal of these compounds in an actively growing plant would be equally rapid. Thus the curve representing their presence at any one stage would be expected to show a low and fairly constant percentage.

Table IX. *Distribution of nitrogen as percentage of total nitrogen.*

	Age (days)	Total nitrogen %	N as NH_3 %	N as nitrate	N as amide	N as protein	N as proteose	N as amino-compounds	N as asparagine	Insol. N	Total
X.	0	6·42	0	0	0	59·2	0	0	0	41·1	100·3
B.	5	9·89	0·26	—	—	—	—	—	—	43·9	—
A.	8	8·24	1·02	—	3·15	20·2	0·85	0·92	21·2	—	—
F.	13	8·7	0·93	3·1	2·76	23·4	1·72	0·95	22·3	41·5	96·6
C.	18	19·16	0·22	2·9	1·41	9·81	14·2	0·29	53·7	20·7	103·2
G.	22	16·29	0·41	5·03	1·41	27·8	10·3	0·25	29·4	24·9	99·4
E.	25	14·56	0·38	2·06	1·65	12·0	3·7	0·30	20·5	43·5	84·2
D.	31	14·49	0·19	3·36	1·34	15·0	—	0·17	14·6	38·9	—
H.	82	12·64	0·37	6·01	1·11	18·5	13·1	0·13	10·6	26·3	—

The rôle of asparagine in the metabolism of the leguminous seed has been investigated by several workers. Schulze [1880, 1911; Schulze and Winterstein, 1910] have noticed its formation in considerable quantity concomitantly with the breakdown of protein on germination. They suggest that its removal corresponds to a re-synthesis of protein, but this has not, so far, received quantitative treatment. Chibnall [1924], in his examination of the mature leaf of the runner bean, finds the same phenomenon and suggests that asparagine is a translocation product whereby nitrogen is conveyed from regions of protein catabolism to those of re-synthesis. The close relationship, shown by the curves, Fig. 1, between the fall of asparagine and proteose and protein re-synthesis in the lupin seedling is striking confirmation of this.

The seed, during the rapid degradation of reserve protein, concentrates its labile nitrogen in the form of the soluble, and hence mobile, compound asparagine. When the peak of catabolic activity is passed, and the young plant has energy free for the building up of its own material, asparagine is quickly removed by further chemical change, and synthesis of proteose and protein begins, passing presumably through the stages of amide, nitrate and ammonium salt.

Summary.

1. Insoluble nitrogenous material, protein and asparagine, represent the bulk of the total nitrogen of the lupin seedling in the seed and in the early stages of growth.

2. A close relationship between decrease of insoluble and protein nitrogen and increase of asparagine is observed.

3. Protein synthesis begins after 16 days' growth and is accompanied by a fall in asparagine content and a rise in proteose content.

4. Ammonia, amides, amino-acids and nitrates are absent from the ungerminated seed. They appear immediately growth begins, rise to a low maximum in a few days and then maintain a constant lower level.

REFERENCES.

Chibnall (1922). *Biochem. J.* **16**, 344.
—— (1924). *Biochem. J.* **18**, 395.
Robinson (1929). *Biochem. J.* **23**, 1099.
Sachsse (1873). *J. prakt. Chem.* **6**, 118.
Schulze (1880). *Landes. Jahrb.* **9**, 689.
—— (1911). *Z. physiol. Chem.* **71**, 31.
—— and Winterstein (1910). *Z. physiol. Chem.* **65**, 431.
Wasteneys and Borsook (1917). *J. Biol. Chem.* **29**, 25.

CCXXXVI. THE BASIC AMINO-ACIDS OF WOOL.

By A. MALCOLM STEWART and CLAUDE RIMINGTON.

From the Wool Industries Research Association, Headingley, Leeds.

(*Received August 3rd, 1931.*)

Only two determinations have been made of the basic amino-acids present in wool, Marston [1928] using the indirect Van Slyke method found a constant proportion of basic nitrogen in several samples of wool analysed. His figures for histidine, arginine and lysine were 6·9, 10·2 and 2·8 % respectively. Vickery and Block [1930], in a careful series of determinations by direct isolation from a sample of wool, found 7·8 % of arginine, 2·3 % of lysine and only 0·66 % of histidine. In their paper they comment upon the fact that other keratins such as human hair have been found to contain about 0·5 % of histidine [Vickery and Leavenworth, 1929], whereas Marston's figures are over ten times as great.

Marston's figures were reported with some reservation, owing to the well-known difficulty of obtaining trustworthy results by the Van Slyke method, particularly when the protein under examination is one containing a large proportion of cystine.

With regard to the amount of arginine in wool, Marston mentioned that "the estimation of arginine by silver precipitation and by gravimetric means utilising flavianic acid as a precipitating agent yielded figures which were quite constant and at considerable variance with the figures yielded by the above methods" (Van Slyke determinations).

In order to clear up the discrepancy, it was thought advisable to verify Vickery and Block's results by a repetition of the determinations using the modified procedure described by Calvery [1929] for the quantitative isolation of the basic amino-acids from small quantities of protein (5 g.).

In the present investigations a single sample of wool has been used, successive duplicate determinations by the Calvery method being carried out and also a direct colorimetric determination of histidine by the Hanke and Koessler [1920] procedure.

The figures obtained by both methods were in close agreement, averaging 0·5 %, and thus confirming those of Vickery's analyses of wool and of human hair.

Experimental.

A fleece from a West Australian 2 years old Merino ewe was scoured by warm steeping in soap and water, thoroughly washed with liberal quantities of water in a hydro-extractor and finally run through a small carding machine

which not only removed much foreign material but also tended to mix the sample. After hand-carding it was picked free from any residual vegetable matter *etc.*

From the 250 g. of wool so prepared, 1 g. samples (duplicate) were taken for ash and 0·1 g. portions (triplicate) for total nitrogen determinations.

Moisture determinations were carried out upon 1 g. samples dried to constant weight at 105° in a stream of dry air.

	Moisture %	Ash %	Total nitrogen* %
	10·66	0·62	16·50
	10·63	0·62	16·43
	—	—	16·37
Mean	10·65	0·62	16·43

* Corrected for ash and moisture.

It was found in a preliminary trial that when 5 g. of wool were boiled with 25 cc. of 20 % hydrochloric acid, maximal amino-nitrogen liberation was attained within 12 hours. There was no change up to 24 hours' boiling, the proportion of nitrogen present in the amino-form being, at this stage, 62·5 % as determined by formaldehyde titration and 82·47 % by reaction with nitrous acid (½ hour in the Van Slyke apparatus).

It is of interest to note that the non-amino-nitrogen calculated on the assumption of 4·4 % of proline, 1·8 % of tryptophan, 7·8 % of arginine, and 0·6 % of histidine works out at 16·23 % in good agreement with the 17·5 % calculated from the Van Slyke figures.

Determination of the bases by means of their silver salts etc.

Hydrolysis was effected by boiling 5 g. wool with 50 cc. of 20 % hydrochloric acid for 18 hours, after which Calvery's procedure was followed. Whenever possible loss of material was guarded against by testing washings of precipitates, crude fractions *etc.*, by means of the appropriate colour reactions—Sakaguchi's test for arginine, the diazo-reaction for histidine and the ninhydrin colour test for amino-acids in solution.

In this way the importance was realised, as emphasised by Calvery, of keeping a strongly acid reaction whilst adding silver oxide and in making distinctly alkaline to phenolphthalein when precipitating the arginine silver salt.

Some inconvenience was caused by the occasional precipitation of silver sulphide arising from the partial decomposition of some cystine in the presence of silver hydroxide. It was removed by filtration.

Of the crude histidine fraction, 50 cc. in all, 25 cc. were taken for total nitrogen, 24 cc. for isolation of the flavianate and 1 cc. diluted to 10 cc., of which 1 cc. aliquots were used for colorimetric histidine determinations by the Hanke and Koessler method.

The Congo red-methyl orange standard was checked against pure histidine hydrochloride and was entirely satisfactory.

As noticed by the other workers, the figures for total nitrogen of the purified fractions were in excess of the quantities of the bases actually isolated. The impurity in the first lysine fraction was particularly large.

Results of determinations.

	Histidine (%)		
Exp. No.	By total nitrogen	By flavianate	By colorimetric determination
1	0·736	0·59	0·54
2	0·733	0·62	0·51

	Arginine (%)	
Exp. No.	By total nitrogen	By flavianate
1	6·64	5·60
2	6·0	5·97

	Lysine (%)	
Exp. No.	By total nitrogen	By picrate
1	7·23	(1·86)*
2	2·24	2·8

* Portion lost.

Histidine by colorimetric determination.

2·5 g. of wool were boiled for 24 hours with 50 cc. of 20 % hydrochloric acid. The hydrolysate was made up to 100 cc. and total nitrogen determined on 2 cc. aliquots. The procedure outlined by Hanke and Koessler was then followed exactly, the final solution of basic phosphotungstate being diluted to 500 cc. Colorimetric determination was carried out upon 1 cc. samples.

Results.

	Histidine
	0·55 %
	0·55 %
Mean	0·55 %

As there was only 0·04 g. of histidine flavianate available for isolation, the values given by the colorimetric method are probably the more reliable.

Discussion.

No claim is made that the proportions of the basic amino-acids present do not vary from wool to wool, since variations as great as 18 % are known to occur in the cystine content of Australian Merino wool [Barritt and King, 1929]. It is clear however that the percentage of histidine is of the order found by Vickery and Block; namely, 0·5–0·6 %.

Since, up to the present, all attempts to discover variability in the amino-acid content of different wools have been confined to cystine alone, the

realisation is important that this variation may well be influenced by variation in any other amino-acid present in the keratin. It would be of interest to carry out a comparative series of determinations of histidine and other easily determined amino-acids in a series of wools of different origin. Constancy of composition is hardly to be expected, nor is it required by the hypothesis put forward by Astbury and Woods [1931] as to the nature of the linkage of amino-acids in wools.

SUMMARY.

1. The basic amino-acids in a fleece of Australian wool have been estimated by Calvery's method. The values for histidine have been verified by Hanke and Koessler's colorimetric method.

2. This wool gave 0·55 % histidine, 6 % arginine and 2·2 % lysine.

3. These figures are in agreement with those given by Vickery and his co-workers for wool and for human hair and support his opinion "that Marston has been led to an erroneous conclusion with respect to the proportion of histidine yielded by wool."

A. M. S. of the University of Western Australia wishes to thank the Council and Director of the Wool Industries Research Association for facilities granted him for working temporarily in their laboratories.

REFERENCES.

Astbury and Woods (1931). *Phil. Trans. Roy. Soc. Lond.* A **230**, 75.
Barritt and King (1929). *J. Text. Inst.* **20**, 151 T.
Calvery (1929). *J. Biol. Chem.* **83**, 631.
Hanke and Koessler (1920). *J. Biol. Chem.* **43**, 527.
Marston (1928). Council Sci. and Ind. Research, Commonwealth of Australia, Bull. 38.
Vickery and Block (1930). *J. Biol. Chem.* **86**, 107.
—— and Leavenworth (1929). *J. Biol. Chem.* **83**, 523.

INDEX

Absorption of water by gelatin (Jordan-Lloyd) 1580; (Pleass) 1943
Accessory factor necessary for growth of *Nematospora gossypii* (Buston and Pramanik) 1656, 1671
Acid-denaturation of haemoglobin (Meldrum) 1498
Acidosis, chlorides and inorganic constituents of serum and cerebrospinal fluid in (Linder and Carmichael) 1090
Adrenaline, effect of, on glycogen distribution in the rat (Lawrence and McCance) 570
Ågren, G., Wilander, O. and Jorpes, E. Cyclic changes in the glycogen content of the liver and the muscles of rats and mice. Their bearing upon the sensitivity of the animals to insulin, and their influence on the urinary output of nitrogen 777
Ahmad, B. The fate of carotene after absorption in the animal organism 1195
Aitken, H. A. A. A further note on the determination of iodine in blood 446
Alcock, R. S., *see* Cook, R. P.
Alcohols, secondary, paraffins and ketones, synthesis and crystal spacings of long-chain (Piper, Chibnall, Hopkins, Pollard, Smith and Williams) 2072
Algae, marine, occurrence of sugar alcohols (dulcitol) in (Haas and Hill) 1470
Allchorne, E., *see* Bacharach, A. L.
Allen, L. A. Observations on some alcohol-soluble proteins from milk products 1045
Aluminium, assimilation of, by the human system (Lewis) 2162
Aluminium, excretion and absorption of, in the rat (Mackenzie) 287
Aluminium in aluminium protein compounds, a method for determining (Pope) 1949
Amino-acids, basic, of wool (Stewart and Rimington) 2189
Amino-acids, Erlenmeyer synthesis of (Lamb and Robson) 1231
Ammonia, oxidation of, in percolating filters (Jenkins) 147
Ampholytes, effect of addition of salts on p_H of solutions of (Giedroyć and Przyłęcki) 465
Amylase and lactic acid formation in muscle, relation between (Case) 561
Amylase-starch-gelatin system (Freiberger) 705
Amylase-starch-protein systems, action of salts on (Chrempińska) 1555
Anslow, W. K. and Raistrick, H. Studies in the biochemistry of micro-organisms. XIX. 6-Hydroxy-2-methylbenzoic acid, a product of the metabolism of glucose by *Penicillium griseo-fulvum* Dierckx 39
Anthocyanin of *Oxycoccus macrocarpus* Pers. (Grove and Robinson) 1706
Anthocyanins, survey of (Robinson and Robinson) 1687
Antiglyoxalase (Giršavičius) 1807
Antimony trichloride test of liver oils (Gillam and Morton) 1346
Antiscorbutic factor, reprecipitation of (Zilva) 594
Antiscorbutic potency of apples (Bracewell, Kidd, West and Zilva) 138; (Bracewell, Wallace and Zilva) 144
Apple cuticle, wax constituents of (Chibnall, Piper, Pollard, Smith and Williams) 2095
Apple, determination of reducing sugars in, iodimetrically (Archbold and Widdowson) 101
Apples, antiscorbutic potency of (Bracewell, Kidd, West and Zilva) 138; (Bracewell, Wallace and Zilva) 144
Archbold, H. K. and Widdowson, E. M. Iodimetric determination of reducing sugars in the apple 101
Arginine, effect of, on body weight of tumour-bearing mice injected with thyroxine (Gilroy) 414
Arsenate, effect of, on the induction period of zymin (Macfarlane) 822
Ashford, C. A. and Holmes, E. G. Further observations on the oxidation of lactic acid by brain tissue 2028
Aspergillus, production of mannitol from hexoses and pentoses by white species of (Coyne and Raistrick) 1513
Auchinachie, D. W., *see* McGowan, J. P.
Auden, H. A. and Dawson, E. R. The hydrolysis of concentrated sugar solutions by invertase 1909
Autolysis in malignant and normal rabbit tissues (Price) 1491
Avitaminosis B, intoxication by methylglyoxal in (Vogt-Møller) 418

Bacharach, A. L., Allchorne, E. and Hazley, V. The effect of adding vitamin A to a rachitogenic diet 639
B. coli cultures, effect of bacteriophage on oxidation-reduction potentials of (Hewitt) 1641
B. coli, effect of hydrogen ion concentration on oxidations by (Cook and Alcock) 523
B. coli, reduction of nitrates by (Stickland) 1543
B. coli, respiration of (Cook and Haldane) 880
B. coli, respiratory catalysts of, relationship between (Cook, Haldane and Mapson) 534
B. dysenteriae cultures, effect of bacteriophage on oxidation-reduction potentials of (Hewitt) 1447
B. pyocyaneus, action of, on citric acid (Walker, Subramaniam, Stent and Butterworth) 129
Bacteria, liberation of elementary nitrogen by (Barritt) 1965
Bacteria, "resting" (Sandiford and Wooldridge) 2172

Bacteria, types of products formed from glucose by (Birkinshaw, Charles and Clutterbuck) 1522
Bacterial proteases, formation of, in synthetic media (Haines) 1851
Bacteriophage, effect of, on oxidation-reduction potentials of *B. coli communis* cultures (Hewitt) 1641
Bacteriophage, effect of, on oxidation-reduction potentials of *B. dysenteriae* (Shiga) cultures (Hewitt) 1447
Bacteriophage, effect of, on oxidation-reduction potentials of *Staphylococcus* cultures (Hewitt) 2068
Banks, A. and Hilditch, T. P. The glyceride structure of beef tallows 1168
Barger, G. and Weichselbaum, T. E. A new synthesis of methionine 997
Barium, micro-determination of (McLaughlin) 307
Barritt, J. and King, A. T. Note on the sulphur-free nature of medulla in blackface wool 1075
Barritt, J. and Rimington, C. The relation between total sulphur and cystine content of certain samples of rabbit fur 1072
Barritt, N. W. The biological filtration of dilute sucrose solutions 1419
Barritt, N. W. The liberation of elementary nitrogen by bacteria 1965
Barton-Wright, E. C. and Boswell, J. G. The biochemistry of dry-rot in wood. II. An investigation of the products of decay of spruce wood rotted by *Merulius lachrymans* 494
Basic amino-acids of wool (Stewart and Rimington) 2189
Bekker, J. G. and King, A. T. Sulphur distribution in the component structures of wool and porcupine quills 1077
Bell, M. E. The blood-sugar level in vitamin B_1 deficiency 1755
Beriberi, experimental, glyoxalase-co-enzyme ratio in (Vogt-Møller) 418
Bhattacharya, B. and Hilditch, T. P. The body fats of the pig. I. Influence of ingested fat on the component fatty acids 1954
Biological values of proteins of various breads (Kon and Markuze) 1476
"Bios," relation of accessory factor for growth of *Nematospora gossypii* to (Buston and Pramanik) 1671
Birch, T. W. and Guha, B. C. The chemical nature of vitamin B_1 from evidence afforded by its electrical transference 1391
Bird, G. M. and Haas, P. On the nature of the cell wall constituents of *Laminaria* spp. Mannuronic acid 403
Birkinshaw, J. H., Callow, R. K. and Fischmann, C. F. Studies in the biochemistry of micro-organisms. XXII. The isolation and characterisation of ergosterol from *Penicillium puberulum* Bainier, grown on a synthetic medium with glucose as sole source of carbon 1977
Birkinshaw, J. H., Charles, J. H. V. and Clutterbuck, P. W. Studies in the biochemistry of micro-organisms. XXI. Quantitative examination by the carbon balance sheet method of the types of products formed from glucose by species of bacteria 1522
Bladder, urinary, phosphatase activity of transplants of (Huggins) 728
Blissett, A. H. and Golding, J. On the inadequacy of white wheat flour and fish meal for prolonged growth of pigs and its amelioration with yeast or stout 349
Blood-calcium following intravenous injection of calcium salts (Greville) 1931
Blood corpuscles, red, quantity of free water in (Schiødt) 8
Blood, determination of inorganic sulphate in (Cuthbertson and Tompsett) 1237
Blood, determination of iodine in (Aitken) 446
Blood, ergothioneine content of (Salt) 1712
Blood, factors influencing complement in (Osborn) 2136
Blood-fat, determination of (Himwich, Friedman and Spiers) 1839
Blood glycolysis (Reid and Narayana) 339
Blood-phosphatases (Roche) 1724
Blood-phosphorus in fat absorption (Vahlquist) 1628
Blood-plasma, human, determination of inorganic sulphate in (Cope) 1183
Blood-sugar, determination of, by the Hagedorn-Jensen method (Kramer and Steiner) 161
Blood-sugar in vitamin B_1 deficiency (Bell) 1755
Blow-fly larvae, collagenase from (Hobson) 1458
Boas-Fixsen, M. A. The effect of desiccation upon the nutritive properties of egg-white. II 596
Booth, J., *see* Campbell, W. G.
Boswell, J. G., *see* Barton-Wright, E. C.
Boxwood, proximate analysis and nature of furfuraldehyde-yielding constituents of (Preece) 1304
Boyd, W. J. The nature of the "ether reaction" of urine 812
Bracewell, M. F., Kidd, F., West, C. and Zilva, S. S. The antiscorbutic potency of apples. II 138
Bracewell, M. F., Wallace, T. and Zilva, S. S. The antiscorbutic potency of apples. III 144
Bracewell, M. F. and Zilva, S. S. Vitamin C in the orange and the grape fruit 1081
Brain tissue, oxidation of lactic acid in (Ashford and Holmes) 2028
Briggs, G. E. The significance of the Michaelis constant for saccharase 1801
Bruins, H. R., Overhoff, J. and Wolff, L. K. The molecular weight of vitamin A 430
Buston, H. W. and Pramanik, B. N. The accessory factor necessary for the growth of *Nematospora gossypii*. I. The chemical nature of the accessory factor 1656
Buston, H. W. and Pramanik, B. N. The accessory factor necessary for the growth of *Nematospora gossypii*. II. The relation of the accessory factor to "bios" 1671
Butter-fats, glyceride structure of (Hilditch and Sleightholme) 507
Butterworth, J., *see* Walker, T. K.

Cabbages, lipoid matter extracted from (Collison and Smedley-MacLean) 606
Calcification of the bones of rats on a diet low in ergosterol (Hume and Smith) 292
Calcium, effect of, on rate of diffusion of sugars through surviving intestine (Magee and Sen) 643
Calcium metabolism of guinea-pigs in scurvy (Humphreys and Zilva) 579
Calcium-phosphate intake, influence of, on hypervitaminosis D (Harris and Innes) 367
Calcium salts, blood-calcium following intravenous injection of (Greville) 1931
CALLOW, R. K. The purification of ergosterol 79
CALLOW, R. K. Occurrence of α-dihydroergosterol as an impurity in yeast ergosterol 87
CALLOW, R. K. and FISCHMANN, C. F. The occurrence of vitamin D in lampreys (Petromyzontidae) 1464
CALLOW, R. K. *see also* BIRKINSHAW, J. H. and ROSENHEIM, O.
CAMPBELL, W. G. The chemistry of the white rots of wood. II. The effect on wood substance of *Armillaria mellea* (Vahl.) Fr., *Polyporus hispidus* (Bull.) Fr., and *Stereum hirsutum* Fr. 2023
CAMPBELL, W. G. and BOOTH, J. The chemical aspect of the drying of timber. 2. The drying of a softwood 756
Cancer tissue, inhibitory effect of monoiodoacetic acid on lactic acid production by (Harrison and Mellanby) 770
CAPPER, N. S., MCKIBBIN, I. M. W. and PRENTICE, J. H. Carotene and vitamin A. The conversion of carotene into vitamin A by fowl 265
Carbohydrate complex of serum-proteins (Rimington) 1062
Carbohydrate content of isolated frog muscle (Kerly) 671
Carbohydrate-free diet, metabolism of animals on (Hynd and Rotter) 1893
Carbohydrate-free diets, sensitivity of animals on, towards insulin (Hynd and Rotter) 457
Carbohydrate, galactose as the dietary (Guha) 1385
Carbohydrate metabolism in birds (Fisher) 1410
Carbohydrates in the sweet potato, chemical changes in, during cooking (Sinoda, Kodera and Oya) 1973
CARMICHAEL, E. A., *see* LINDER, G. C.
Carotene, conversion of, into vitamin A by fowl (Capper, McKibbin and Prentice) 265
Carotene, distribution of, in the body of the rat (Moore) 275
Carotene, fate of, after absorption in the animal organism (Ahmad) 1195
Carotene, vitamin A and (Moore) 2131
CASE, E. M. The relation between amylase and lactic acid formation in muscle 561
CASE, E. M. and COOK, R. P. The occurrence of pyruvic acid and methylglyoxal in muscle metabolism 1319
CASE, E. M., *see also* MCCULLAGH, D. R.
Caseinogen, effect of proteinase of green malt on (Hopkins and Kelly) 256
Caseinogen for vitamin tests (Coward, Key, Dyer and Morgan) 551
Catabolism, increased, distribution of nitrogen and sulphur in urine during Cuthbertson) 236
Catalase, effect of, on oxidation-reduction potentials of haemolytic streptococcus cultures (Hewitt) 858
Catalase, effect of, on oxidation-reduction potentials of pneumococcus cultures (Hewitt) 169
Catalysis by copper sulphate of oxidation of oleic acid by hydrogen peroxide (Smedley-MacLean and Pearce) 1252
Catalysis of oxidation of cysteine and glutathione by iron and copper (Pirie) 1565
Cereals, vitamin B_1 values of (Plimmer, Raymond and Lowndes) 691
Cerebrospinal fluid, chlorides and inorganic constituents of, in nephritis and acidosis (Linder and Carmichael) 1090
CHARLES, J. H. V., *see* BIRKINSHAW, J. H.
CHIBNALL, A. C., PIPER, S. H., POLLARD, A., SMITH, J. A. B. and WILLIAMS, E. F. The wax constituents of the apple cuticle 2095
CHIBNALL, A. C., *see also* PIPER, S. H. and POLLARD, A.
Chick, fat-soluble vitamin requirements of (McFarlane, Graham and Richardson) 358
Cholesterol and phosphatide contents of normal and malignant human tissues (Jowett) 1991
Cholesterol and phosphatides, determination of small amounts of (Jowett and Lawson) 1981
CHREMPIŃSKA, H. Structure and enzyme reactions. X. Action of salts on the systems: amylase-starch-proteins 1555
Chromogenic constituents of fresh and aged liver oils (Heilbron, Gillam and Morton) 1352
Citric acid, action of *B. pyocyaneus* on (Walker, Subramaniam, Stent and Butterworth) 129
Citric acid dehydrogenase of cucumber seeds, temperature coefficients and energy exchanges of (Dann) 177
CLIFFORD, W. M. The loss of glucose from dried peas on soaking 1999
CLUTTERBUCK, P. W., *see* BIRKINSHAW, J. H.
Cod-liver oils, determination of vitamin A in (Coward, Dyer, Morton and Gaddum) 1102
COLE, S. W. A method for the direct determination of urea in urine 1653
Collagenase from blow-fly larvae (Hobson) 1458
COLLIN, G. Some peculiarities in the glyceride structure of laurel fats 95
COLLISON, D. L. and SMEDLEY-MACLEAN, I. The nature of the lipoid matter extracted from green leaves (spinach and cabbage) 606
Colloids, effect of p_H on precipitating and protective actions of mixtures of proteins on (Hopkins, Kermack and Spragg) 829, 1860
Complement in the blood, factors influencing (Osborn) 2136
COOK, R. P. and ALCOCK, R. S. The effect of hydrogen ion concentration on some oxidations by *B. coli communis* 523

Cook, R. P. and Haldane, J. B. S. The respiration of *B. coli communis* 880
Cook, R. P., Haldane, J. B. S. and Mapson, L. W. The relationship between the respiratory catalysts of *B. coli* 534
Cook, R. P., *see also* Case, R. M.
Cooking, chemistry of (Sinoda, Kodera and Oya) 1973
Cope, C. L. Determination of inorganic sulphate in human blood-plasma by microtitration 1183
Copper in animal nutrition (Cunningham) 1267
Coprophagy, effects of, in rats deprived of vitamin B complex (Roscoe) 2056
Coward, K. H., Dyer, F. J., Morton, R. A. and Gaddum, J. H. The determination of vitamin A in cod-liver oils (*a*) biologically, (*b*) chemically, (*c*) physically, with a statistical examination of the results 1102
Coward, K. H., Key, K. M., Dyer, F. J. and Morgan, B. G. E. Caseinogen for vitamin tests 551
Coyne, F. P. and Raistrick, H. Studies in the biochemistry of micro-organisms. XX. On the production of mannitol from hexoses and pentoses by a white species of *Aspergillus* 1513
Co-zymase, activation of oxido-reductions by (v. Euler and Nilsson) 2168
Creed, R. H., *see* Lovern, J. A.
Cunningham, I. J. Some biochemical and physiological aspects of copper in animal nutrition 1267
Cunningham, I. J., *see also* McGowan, J. P.
Cuprous derivatives of some sulphydryl compounds (Pirie) 614
Cuthbertson, D. P. The distribution of nitrogen and sulphur in the urine during conditions of increased catabolism 236
Cuthbertson, D. P. and Tompsett, S. L. A preliminary note on the inorganic sulphate content of the blood with a method for its determination 1237
Cuthbertson, D. P. and Tompsett, S. L. The enzymic synthesis of proteins with special reference to the action of pepsin 2004
Cyanide and other salts, effects of, on fermentation by yeast preparations (Patterson) 1593
Cysteine, catalysis of oxidation of, by iron and copper (Pirie) 1565
Cystine and total sulphur of rabbit fur (Barritt and Rimington) 1072
Cystine, determination of, in proteins (Tompsett) 2014

Damodaran, M. The amino-acids of glutenin 190
Damodaran, M. The dicarboxylic acid-nitrogen of proteins. With a note on the alcohol-soluble protein from ragi (*Eleusine coracana*) 2123
Dann, W. J. The temperature coefficients and energy exchanges of the citric acid dehydrogenase of cucumber seeds 177
Dann, W. J. and Moore, T. The effect of vitamin B deficiency upon the vitamin A reserves of the rat 914
Dawson, E. R., *see* Auden, H. A.
Dehydrogenase, citric acid, of cucumber seeds, temperature coefficients and energy exchanges of (Dann) 177
Dehydrogenases, effect of narcotics on (Sen) 849
Diastase, malt, effect of proteins and protein derivatives on hydrolysis of starch by (Filipowicz) 1874
Dickens, F. and Šimer, F. The metabolism of normal and tumour tissue. III. A method for the measurement of respiratory quotient in serum 973
Dickens, F. and Šimer, F. The metabolism of normal and tumour tissue. IV. The respiratory quotient in bicarbonate-media 985
Diet, rachitogenic, effect of addition of vitamin A to (Bacharach, Allchorne and Hazley) 639
Dietary factors, rôle of, in formation of erythrocytes (Guha and Mapson) 1674
α-Dihydroergosterol, occurrence of, as an impurity in yeast ergosterol (Callow) 87
Dihydroxyacetone (Reeves and Renbom) 412
3 : 4-Dihydroxyphenylalanines, synthesis of the optically active (Harington and Randall) 1028
d-3 : 5-Diiodotyrosine, isolation of, from the thyroid gland by the action of proteolytic enzymes (Harington and Randall) 1032
Dissociation constants of *i*-*β*-hydroxyglutamic acid (Harington, Randall and Richardson) 1917
Dry-rot in wood, biochemistry of (Barton-Wright and Boswell) 494
Dudley, H. W. Intermediary carbohydrate metabolism. The effect of sodium iodoacetate on glyoxalase 439
Dulcitol, occurrence of, in marine algae (Haas and Hill) 1470
Dunlop, D. M., *see* Stewart, C. P.
Dyer, F. J., *see* Coward, K. H.
Dyestuffs, effect of, on enzymes of oxidation (Quastel and Wheatley) 629
Dyestuffs, effect of, on fumarase (Quastel) 898

Edestin, effect of proteinase of green malt on (Hopkins and Kelly) 256
Egg-albumin, effect of proteinase of green malt on (Hopkins) 245
Egg-white, effect of desiccation upon the nutritive properties of (Boas-Fixsen) 596
Electrodes, "indifferent," behaviour of, in determination of oxidation-reduction potentials in presence of hydrogen (Lepper and Martin) 45
Ellis, R. W. B., *see* Goldblatt, M. W.
Elphick, G. K., *see* Key, K. M.
Enterokinase, effect of heat upon the activating efficiency of (Pace) 1
Enzyme action, the addition compound theory of (Woolf) 342
Enzyme from animal tissues oxidising hexose-diphosphoric acid (Harrison) 1011
Enzyme from blow-fly larvae which digests collagen in alkaline solution (Hobson) 1458
Enzyme, oxidising, from animal tissues (Harrison) 1016
Enzyme reactions, structure and (Freiberger) 705; (Chrempińska) 1555

Enzymes, effect of dyestuffs on (Quastel and Wheatley) 629
Enzymes, trypanocidal action and toxicity to (Quastel) 1121
Enzymic synthesis of proteins with special reference to the action of pepsin (Cuthbertson and Tompsett) 2004
Epidermis, human, permeability of, to ultra-violet irradiation (Lucas) 57
Ergosterol, calcification of the bones of rats on a diet low in (Hume and Smith) 292
Ergosterol, isolation and characterisation of, from *Penicillium puberulum* Bainier, grown on a synthetic medium with glucose as sole source of carbon (Birkinshaw, Callow and Fischmann) 1977
Ergosterol, purification of (Callow) 79
Ergothioneine content of the blood in health and disease (Salt) 1712
Erlenmeyer synthesis of amino-acids (Lamb and Robson) 1231
Erythrocytes, rôle of dietary factors in formation of (Guha and Mapson) 1674
Erythrocytes, sensitisation of, in haemolysis (Sen) 1244
Esterase, liver, inhibitory action of synthetic urethanes on (Stedman and Stedman) 1147
"Ether reaction" of urine, the (Boyd) 812
EULER, H. v. and NILSSON, R. Note on the activation of oxido-reductions by co-zymase 2168
Excretion, nitrogen, effect of insulin on (Goldblatt and Ellis) 221
Exercise, muscular, fat metabolism in (Stewart, Gaddie and Dunlop) 733
EYRE, J. V. Notes on oil development in the seed of a growing plant 1902

Fat absorption, blood-phosphorus in (Vahlquist) 1628
Fat-free diet, relation of, to scaly tail condition in rats (Hume and Smith) 300
Fat metabolism in muscular exercise (Stewart, Gaddie and Dunlop) 733
Fats, body, of the pig. I. Influence of ingested fat (Bhattacharya and Hilditch) 1954
Fats, laurel, peculiarities in the glyceride structure of (Collin) 95
Fats, natural, spectrographic data of, in relation to vitamin A (Gillam, Heilbron, Hilditch and Morton) 30
Fatty acids of cat's kidney (Turner) 49
Fatty acids of natural fats, spectrographic data of, in relation to vitamin A (Gillam, Heilbron, Hilditch and Morton) 30
Fermentation by yeast preparations, effects of cyanide and other salts on (Patterson) 1593
Fermentation, selective, of glucose and fructose (Hopkins) 245
Fibres, outer-coat animal, relation between cystine yield and total sulphur in (Rimington) 71
Fibrin, effect of proteinase of green malt on (Hopkins and Kelly) 256
FILIPOWICZ, B. Influence of proteins and protein derivatives on the enzymic hydrolysis of starch by malt diastase 1874
Filters, percolating, biological oxidation of sucrose and ammonia in (Jenkins) 147
Filtration, biological, of dilute sucrose solutions (Barritt) 1419
FINE, J. On the influence of serum on enzymes, with special reference to its action on trypsin 647
FISCHMANN, C. F., *see* BIRKINSHAW, J. H. and CALLOW, R. K.
Fish meal, inadequacy of, for prolonged growth of pigs (Blissett and Golding) 349
Fish meal, vitamin A and vitamin D content of (McFarlane, Graham and Richardson) 358
FISHER, R. B. Carbohydrate metabolism in birds. III. The effects of rest and exercise upon the lactic acid content of the organs of normal and rice-fed pigeons 1410
Fish-livers, Mittelmann process for treating (Lovern, Creed and Morton) 1341
FREIBERGER, S. Structure and enzyme reactions. IX. The systems amylase-starch-gelatin and urease-urea-gelatin 705
FRIEDMAN, H., *see* HIMWICH, H. E.
Fructose, effect of, on ammonia and urea production of embryo kidney tissue *in vitro* (Watchorn and Holmes) 843
Fruits and vegetables, vitamin B_1 values of (Plimmer, Raymond and Lowndes) 1788
Fruits, distribution of vitamin B complex in (Roscoe) 2050
Fumarase, action of dyestuffs on (Quastel) 898
Fur, rabbit, relation between total sulphur and cystine content of (Barritt and Rimington) 1072

GADDIE, R., *see* STEWART, C. P.
GADDUM, J. H., *see* COWARD, K. H.
Galactose as the dietary carbohydrate (Guha) 1385
Galactose, effect of, on ammonia and urea production of embryo kidney tissue *in vitro* (Watchorn and Holmes) 843
Gallotannin, action of tannase on (Nicholson, Nierenstein, Pool and Price) 752
GAVRILESCU, N. The iodine method for the determination of glutathione 1190
GAVRILESCU, N. and PETERS, R. A. Biochemical lesions in vitamin B deficiency 1397
GAVRILESCU, N. and PETERS, R. A. On the function of torulin. An *in vitro* effect of antineuritic vitamin concentrates 2150
Gelatin, absorption of water by (Jordan-Lloyd) 1580
Gelatin, absorption of water by; combination of gelatin with acids (Pleass) 1943
Gelatin and ovalbumin, electrophoretic velocities of, in their mixtures (Prideaux and Howitt) 391
GIEDROYĆ, G. and PRZYŁĘCKI, S. J. Effect of the addition of salts on the p_H of solutions of ampholytes 465
GIEDROYĆ, W., *see also* PRZYŁĘCKI, S. J.
GILLAM, A. E., HEILBRON, I. M., HILDITCH, T. P. and MORTON, R. A. Spectrographic data of natural fats and their fatty acids in relation to vitamin A 30
GILLAM, A. E. and MORTON, R. A. The antimony trichloride test and the ultra-violet absorption of liver oils and concentrates 1346

GILLAM, A. E., *see also* HEILBRON, I. M.
GILROY, E. The effect of arginine on the body weight of mice injected with thyroxine, and bearing the tumour M 63 414
GIRŠAVIČIUS, J. O. Studies on antiglyoxalase. II. Methods of following glyoxalase action 1807
Glucosaminodimannose, isolation of, from carbohydrate complex of serum-proteins (Rimington) 1062
Glucose dehydrogenase: a new oxidising enzyme from animal tissues (Harrison) 1016
Glucose, determination of renal threshold for (Himsworth) 1128
Glucose, loss of, from dried peas on soaking (Clifford) 1999
Glucose, types of products formed from, by species of bacteria (Birkinshaw, Charles and Clutterbuck) 1522
Glutamic acid peptide from seaweed (Haas and Hill) 1472
Glutathione, catalysis of oxidation of, by iron and copper (Pirie) 1565
Glutathione, iodine method for determination of (Gavrilescu) 1190
Glutathione, metabolism of, in the dog (Hele and Pirie) 1095
Glutenin, amino-acids of (Damodaran) 190
Glyceride structure of beef tallows (Banks and Hilditch) 1168
Glycogen content of liver and muscles of rats and mice, cyclic changes in, in relation to sensitivity to insulin and urinary output of nitrogen (Ågren, Wilander and Jorpes) 777
Glycogen, distribution of, in the rat, effect of starvation, phloridzin, thyroid, adrenaline, insulin and pituitrin on (Lawrence and McCance) 570
Glycolysis, blood (Reid and Narayana) 339
Glycosuria, relation of, to glycaemia (Himsworth) 1128
Glyoxalase action, methods of following (Giršavičius) 1807
Glyoxalase-co-enzyme ratio in experimental beriberi (Vogt-Møller) 418
Glyoxalase-co-enzyme ratio in liver tissue in inanition (Vogt-Møller) 1540
Glyoxalase, effect of sodium iodoacetate on (Dudley) 439
Gold, colloidal, effect of hydrogen ion concentration on precipitating and protective actions of proteins on (Hopkins, Kermack and Spragg) 829, 1860
GOLDBLATT, M. W. and ELLIS, R. W. B. The effect of insulin on growth, nitrogen excretion and respiratory metabolism 221
GOLDING, J., *see* BLISSETT, A. H.
GRAHAM, W. R., Jr., *see* MCFARLANE, W. D.
Grape fruit, vitamin C in (Bracewell and Zilva) 1081
Grasses, forage, wax constituents of (Pollard, Chibnall and Piper) 2111
GREVILLE, G. D. Study of the blood-calcium following the intravenous injection of calcium salts 1931
GROLLMAN, A. The vapour pressure depressions of aqueous solutions of phosphate buffer mixtures at 20·3° 166
GROVE, K. E. and ROBINSON, R. An anthocyanin of *Oxycoccus macrocarpus* Pers. 1706
Growth and chemical composition of functioning fowl's ovary (Romanoff) 994
Growth, effect of insulin on (Goldblatt and Ellis) 221
GUHA, B. C. Investigations on the preparation and behaviour of vitamin B_1 concentrates from yeast 931
GUHA, B. C. Investigations on vitamin B_2. I. The sources of vitamin B_2. II. The stability of vitamin B_2. III. The chemistry of vitamin B_2 945
GUHA, B. C. Observations on certain factors necessary for the normal nutrition of the rat 960
GUHA, B. C. The physiological function of vitamin B_1 1367
GUHA, B. C. On galactose as the dietary carbohydrate 1385
GUHA, B. C. and MAPSON, L. W. The rôle of certain dietary factors in the formation of erythrocytes 1674
GUHA, B. C., *see also* BIRCH, T. W.
Gum benzoin, effect of hydrogen ion concentration on precipitating and protective actions of proteins on (Hopkins, Kermack and Spragg) 829, 1860

HAAS, P. and HILL, T. G. The occurrence of sugar alcohols in marine algae. Dulcitol 1470
HAAS, P. and HILL, T. G. A preliminary note on the nitrogen metabolism of seaweeds. Glutamic acid peptide 1472
HAAS, P., *see also* BIRD, G. M.
Haemoglobin, acid-denaturation of (Meldrum) 1498
Haemolysis, sensitisation of erythrocytes in (Sen) 1244
Hagedorn-Jensen-Hanes method for determination of reducing sugars, modification of (Hulme and Narain) 1051
HAINES, R. B. The formation of bacterial proteases, especially in synthetic media 1851
HALDANE, J. B. S., *see* COOK, R. P.
HARDEN, A. and MACFARLANE, M. G. Note on the reactivation of reductase in washed yeast preparations 818
HARDING, V. J. and SELBY, D. L. Fermentable sugar in normal urine 1815
HARINGTON, C. R. and RANDALL, S. S. Synthesis of the *d*- and *l*-3 : 4-dihydroxyphenylalanines 1028
HARINGTON, C. R. and RANDALL, S. S. The isolation of *d*-3 : 5-diiodotyrosine from the thyroid gland by the action of proteolytic enzymes 1032
HARINGTON, C. R., RANDALL, S. S. and RICHARDSON, G. M. Synthesis of *i*-β-hydroxyglutamic acid. With a note on the dissociation constants 1917
HARRIS, L. J. and INNES, J. R. M. The mode of action of vitamin D. Studies on hypervitaminosis D. The influence of the calcium-phosphate intake 367
HARRISON, D. C. The oxidation of hexosediphosphoric acid by an enzyme from animal tissues 1011

HARRISON, D. C. Glucose dehydrogenase: a new oxidising enzyme from animal tissues 1016
HARRISON, G. A. Spermine in human tissues 1885
HARRISON, S. T. and MELLANBY, E. A note on the inhibitory effect of monoiodoacetic acid on lactic acid production by cancer tissue 770
HAZLEY, V., *see* BACHARACH, A. L.
Heat-inactivation of proteinase of pancreas (Pace) 1485
Heat-inactivation of trypsin-kinase (Pace) 422
HEILBRON, I. M., GILLAM, A. E. and MORTON, R. A. Specificity in tests for vitamin A. A new conception of the chromogenic constituents of fresh and aged liver oils 1352
HEILBRON, I. M., *see also* GILLAM, A. E. and MORTON, R. A.
HELE, T. S. Studies in the sulphur metabolism of the dog. IX. The synthesis of phenyl sulphate and indoxyl sulphate 1736
HELE, T. S. and PIRIE, N. W. Studies in the sulphur metabolism of the dog. VIII. The metabolism of glutathione compared with that of other cystine derivatives 1095
Hemicelluloses of box-wood (Preece) 1304
Hemicelluloses of the wood of English oak (O'Dwyer) 2017
HEWITT, L. F. Oxidation-reduction potentials of pneumococcus cultures. II. Effect of catalase 169
HEWITT, L. F. Oxidation-reduction potentials of haemolytic streptococci. II. Effect of catalase 858
HEWITT, L. F. Effect of bacteriophage on oxidation-reduction potentials of *B. dysenteriae* (Shiga) cultures 1447
HEWITT, L. F. Effect of lysozyme on the oxidation-reduction potentials of *M. lysodeikticus* cultures 1452
HEWITT, L. F. Effect of bacteriophage on the oxidation-reduction potentials of *B. coli communis* cultures 1641
HEWITT, L. F. Oxidation-reduction potentials of *Staphylococcus* cultures. II. Effect of bacteriophage 2068
Hexosediphosphate, effect of iodoacetic acid on the fermentation of (Macfarlane) 822
Hexosediphosphoric acid, oxidation of, by an enzyme from animal tissues (Harrison) 1011
Hexosemonophosphoric ester, methylation of (King, McLaughlin and Morgan) 310
Hexosemonophosphoric esters (Robison and King) 323
HILDITCH, T. P. and SLEIGHTHOLME, J. J. The glyceride structure of butter-fats 507
HILDITCH, T. P., *see also* BANKS, A. and GILLAM, A. E.
HILL, T. G., *see* HAAS, P.
HIMSWORTH, H. P. The relation of glycosuria to glycaemia and the determination of the renal threshold for glucose 1128
HIMWICH, H. E., FRIEDMAN, H. and SPIERS, M. A. A modification of the method of Stewart and White for the determination of blood-fat, with observations on several species in post-absorptive conditions 1839
HOBSON, R. P. On an enzyme from blow-fly larvae (*Lucilia sericata*) which digests collagen in alkaline solution 1458
HOLMES, B. E., *see* WATCHORN, E.
HOLMES, E. G., *see* ASHFORD, C. A.
HOPKINS, R. H. Selective fermentation. Alcoholic fermentation of mixtures of glucose and fructose by brewer' and Sauterne yeasts 245
HOPKINS, R. H. and KELLY, H. E. The proteolytic enzymes of green malt. II. Action of the proteinase on egg-albumin, caseinogen, edestin and fibrin at different reactions 256
HOPKINS, R. H., KERMACK, W. O. and SPRAGG, W. T. The effect of hydrogen ion concentration on the precipitating and protective actions of proteins on colloidal gold and gum benzoin 829
HOPKINS, R. H., KERMACK, W. O. and SPRAGG, W. T. The effect of hydrogen ion concentration on the precipitating and protective actions of mixtures of two proteins on colloidal gold and gum benzoin 1860
HOPKINS, S. J., *see* PIPER, S. H.
HOWITT, F. O., *see* PRIDEAUX, E. B. R.
HUGGINS, C. B. The phosphatase activity of transplants of the epithelium of the urinary bladder to the abdominal wall producing heterotopic ossification 728
HULME, A. C. and NARAIN, R. The ferricyanide method for the determination of reducing sugars. A modification of the Hagedorn-Jensen-Hanes technique 1051
HUME, E. M. and SMITH, H. H. Calcification of the bones of rats on a diet low in ergosterol 292
HUME, E. M. and SMITH, H. H. The relation of a fat-free diet to the scaly tail condition in rats described by Burr and Burr 300
HUMPHREYS, F. E. and ZILVA, S. S. Metabolism in scurvy. III. The absorption and retention of calcium and phosphorus by guinea-pigs 579
Hydrogenase (Stephenson and Stickland) 205, 215
Hydrogen ion concentration, effect of, on oxidations by *B. coli communis* (Cook and Alcock) 523
Hydrogen ion concentration, effect of, on precipitating and protective actions of proteins on colloidal gold and gum benzoin (Hopkins, Kermack and Spragg) 829, 1860
Hydrogen ion concentration of the potato tuber (Robertson and Smith) 763
i-β-Hydroxyglutamic acid, synthesis and dissociation constants of (Harington, Randall and Richardson) 1917
6-Hydroxy-2-methylbenzoic acid from glucose by the action of *P. griseo-fulvum* (Anslow and Raistrick) 39
Hydroxypyruvic aldehyde (Hynd) 11
HYND, A. Hydroxypyruvic aldehyde: its preparation and physiological behaviour 11
HYND, A. and ROTTER, D. L. Studies on the metabolism of animals on a carbohydrate-free diet. II. Variations in the sensitivity towards insulin of different species of animals on carbohydrate-free diets. 457
HYND, A. and ROTTER, D. L. Studies on the metabolism of animals on a carbohydrate-free diet. III. Effect of alkali on the sensitivity towards insulin of albino rats 1893

Hypervitaminosis D, influence of calcium-phosphate intake on (Harris and Innes) 367

Induction period of zymin, effect of arsenate on (Macfarlane) 822
INNES, J. R. M., *see* HARRIS, L. J.
Insulin, effect of alkali on sensitivity of albino rats to (Hynd and Rotter) 1893
Insulin, effect of, on glycogen distribution in the rat (Lawrence and McCance) 570
Insulin, effect of, on growth, nitrogen excretion and respiratory metabolism (Goldblatt and Ellis) 221
Insulin, sensitivity to, in relation to cyclic changes in glycogen content of liver and muscles of rats and mice (Ågren, Wilander and Jorpes) 777
Insulin, sensitivity to, of animals on carbohydrate-free diets (Hynd and Rotter) 457
Intestine, surviving, influence of calcium on rate of diffusion of sugars through (Magee and Sen) 643
Invertase, hydrolysis of concentrated sugar solutions by (Auden and Dawson) 1909
Iodine, determination of, in blood (Aitken) 446
Iodoacetate, sodium, effect of, on glyoxalase (Dudley) 439
Iodoacetic acid, effect of, on the fermentation of hexosediphosphate (Macfarlane) 822
Iodoacetic acid, inhibitory effect of, on lactic acid production by cancer tissue (Harrison and Mellanby) 770
Iodobenzene and *p*-iodophenylmercapturic acid, metabolism of, in rodents (Lawrie) 1037
Isoelectric point of proteins, effect of salts on (Przyłęcki) 713; (Przyłęcki and Giedroyć) 1734

JENKINS, S. H. The biological oxidation of carbohydrate solutions. Part I. The oxidation of sucrose and ammonia in sectional percolating filters 147
JORDAN-LLOYD, D. The absorption of water by gelatin. VI. The influence of the thickness and original concentration of the gel 1580
JORPES, E., *see* ÅGREN, G.
JOWETT, M. The phosphatide and cholesterol contents of normal and malignant human tissues 1991
JOWETT, M. and LAWSON, E. W. Determination of small amounts of phosphatides and cholesterol in tissues 1981

KELLY, H. E., *see* HOPKINS, R. H.
Kemp, relation between cystine yield and total sulphur in (Rimington) 71
KERLY, M. The total carbohydrate content of isolated frog muscle 671
KERMACK, W. O., *see* HOPKINS, R. H.
Ketones, paraffins and secondary alcohols, synthesis and crystal spacings of long-chain (Piper, Chibnall, Hopkins, Pollard, Smith and Williams) 2072
KEY, K. M. and ELPHICK, G. K. A quantitative method for the determination of vitamin C 888
KEY, K. M., *see also* COWARD, K. H.
KIDD, F., *see* BRACEWELL, M. F.
Kidney tissue, embryo, growing *in vitro*, effect of fructose, galactose and xylose on ammonia and urea production of (Watchorn and Holmes) 843
KING, A. T., *see* BARRITT, J. and BEKKER, J. G.
KING, E. J. The enzymic hydrolysis of lecithin 799
KING, E. J., MCLAUGHLIN, R. R. and MORGAN, W. T. J. The methylation of hexosemonophosphoric ester 310
KING, E. J., *see also* ROBISON, R.
KODERA, S., *see* SINODA, O.
KON, S. K. The self-selection of food constituents by the rat 473
KON, S. K. A study of nitrogen balance in vitamin B_2 deficiency in the rat 482
KON, S. K. and MARKUZE, Z. The biological values of the proteins of breads baked from rye and wheat flours alone or combined with yeast or soya bean flour 1476
KRAMER, H. and STEINER, A. Notes on the Hagedorn-Jensen method for the determination of blood-sugar 161

Lactic acid content of organs of normal and rice-fed pigeons, effects of rest and exercise on (Fisher) 1410
Lactic acid formation in muscle and amylase, relation between (Case) 561
Lactic acid, oxidation of, in brain tissue (Ashford and Holmes) 2028
Lactic acid production by cancer tissue, inhibitory effect of monoiodoacetic acid on (Harrison and Mellanby) 770
LAMB, J. and ROBSON, W. The Erlenmeyer synthesis of amino-acids 1231
Laminaria, cell wall of, nature of constituents of (Bird and Haas) 403
Laminaria, mannuronic acid from (Bird and Haas) 403
Lampreys, occurrence of vitamin D in (Callow and Fischmann) 1464
LAWRENCE, R. D. and MCCANCE, R. A. The effect of starvation, phloridzin, thyroid, adrenaline, insulin and pituitrin on the distribution of glycogen in the rat 570
LAWRIE, N. R. The metabolism of iodobenzene and *p*-iodophenylmercapturic acid in the rat and the rabbit 1037
LAWSON, E. W., *see* JOWETT, M.
Leaves, green, lipoid matter extracted from (Collison and Smedley-MacLean) 606
Lecithin, enzymic hydrolysis of (King) 799
LEPPER, E. H. and MARTIN, C. J. On the behaviour of "indifferent" electrodes when used for the determination of oxidation-reduction potentials in the presence of hydrogen 45
LEWIS, S. J. The assimilation of aluminium by the human system 2162
LINDER, G. C. and CARMICHAEL, E. A. The chlorides and inorganic constituents of the serum and cerebrospinal fluid in nephritis and acidosis 1090
Lipoid matter extracted from green leaves (Collison and Smedley-MacLean) 606
Liver oils, antimony trichloride test and ultra-violet absorption of (Gillam and Morton) 1346

Liver oils, chromogenic constituents of fresh and aged (Heilbron, Gillam and Morton) 1352
Liver oils, spectrographic data concerning (Morton, Heilbron and Thompson) 20
Liver tissue in inanition, glyoxalase-co-enzyme ratio of (Vogt-Møller) 1540
Lovern, J. A., Creed, R. H. and Morton, R. A. The Mittelmann process for treating fish-livers 1341
Lovern, J. A. and Morton, R. A. Pigmentation of the liver of the monk (or angler) fish 1336
Lowndes, J., *see* Plimmer, R. H. A.
Lucas, N. S. The permeability of human epidermis to ultra-violet irradiation 57
Lupin seedling, nitrogen metabolism of (McKie) 2181
Lysozyme, effect of, on oxidation-reduction potentials of *M. lysodeikticus* cultures (Hewitt) 1452

McCance, R. A. and Shipp, H. L. The colorimetric determination of sodium 449
McCance, R. A. and Shipp, H. L. The micro-determination of sodium in biological materials 1845
McCance, R. A., *see also* Lawrence, R. D.
McCullagh, D. R. and Case, E. M. A differential resistance thermometer for biological use 1213
McCullagh, D. R. and Case, E. M. Studies on "non-irritable" muscle 1220
Macfarlane, M. G. Fermentation by yeast preparations. I. The effect of mono-iodoacetate on the fermentation of hexose-diphosphate. II. The action of arsenate on the induction period of zymin 822
Macfarlane, M. G., *see also* Harden, A.
McFarlane, W. D., Graham, W. R., Jr., and Richardson, F. The fat-soluble vitamin requirements of the chick. I. The vitamin A and vitamin D content of fish meal and meat meal 358
McGowan, J. P., Cunningham, I. J., and Auchinachie, D. W. On the fundamental nature of vitamin D action 1295
Mackenzie, K. The biochemistry of aluminium. II. Excretion and absorption of aluminium in the rat 287
McKibbin, I. M. W., *see* Capper, N. S.
McKie, P. The nitrogen metabolism of the lupin seedling 2181
McLaughlin, R. R. The micro-determination of barium 307
McLaughlin, R. R., *see also* King, E. J.
Magee, H. E. and Sen, K. C. The influence of calcium on the rate of diffusion of sugars through surviving intestine 643
Mann, P. J. G. The kinetics of peroxidase action 918
Mannitol, production of, from hexoses and pentoses by white species of *Aspergillus* (Coyne and Raistrick) 1513
Mannuronic acid from *Laminaria* (Bird and Haas) 403
Mapson, L. W., *see* Cook, R. P. and Guha, B. C.
Markuze, Z., *see* Kon, S. K.
Martin, C. J., *see* Lepper, E. H.
Meat meal, vitamin A and vitamin D content of (McFarlane, Graham and Richardson) 358
Meldrum, N. U. The acid-denaturation of haemoglobin 1498
Mellanby, E., *see* Harrison, S. T.
Merulius lachrymans, products of decay of spruce wood rotted by (Barton-Wright and Boswell) 494
Metabolism, calcium and phosphorus of guinea-pigs in scurvy (Humphreys and Zilva) 579
Metabolism, carbohydrate, in birds (Fisher) 1410
Metabolism, carbohydrate, intermediary (Dudley) 439
Metabolism, fat, in muscular exercise (Stewart, Gaddie and Dunlop) 733
Metabolism in tetany (Morris, Watson and Morris) 786
Metabolism, muscle, occurrence of pyruvic acid and methylglyoxal in (Case and Cook) 1319
Metabolism, nitrogen, of seaweeds (Haas and Hill) 1472
Metabolism, nitrogen, of the lupin seedling (McKie) 2181
Metabolism of animals on a carbohydrate-free diet (Hynd and Rotter) 1893
Metabolism of iodobenzene and *p*-iodophenyl-mercapturic acid in rodents (Lawrie) 1037
Metabolism of normal and tumour tissue (Dickens and Šimer) 973, 985
Metabolism, respiratory, effect of insulin on (Goldblatt and Ellis) 221
Metabolism, sulphur, in the dog (Hele and Pirie) 1095; (Hele) 1736
Methionine, synthesis of (Barger and Weichselbaum) 997
Methylglyoxal, occurrence of, in muscle metabolism (Case and Cook) 1319
Michaelis constant for saccharase, significance of (Briggs) 1801
M. lysodeikticus cultures, effect of lysozyme on oxidation-reduction potentials of (Hewitt) 1452
Micro-organisms, biochemistry of (Coyne and Raistrick) 1513; (Birkinshaw, Charles and Clutterbuck) 1522; (Birkinshaw, Callow and Fischmann) 1977
Milk, influence of sunlight on (Whitehead) 1647
Milk products, alcohol-soluble proteins from (Allen) 1045
Mittelmann process for treating fish-livers (Lovern, Creed and Morton) 1341
Monk (or angler) fish, pigmentation of the liver of (Lovern and Morton) 1336
Moore, T. Vitamin A and carotene. VII. The distribution of vitamin A and carotene in the body of the rat 275
Moore, T. Vitamin A and carotene. VIII. High potency vitamin A concentrates 2131
Moore, T., *see also* Dann, W. J.
Morgan, B. G. E., *see* Coward, K. H.
Morgan, W. T. J., *see* King, E. J.
Morris, N., *see* Morris, S.
Morris, S., Watson, A. McL. and Morris, N. Metabolism studies in tetany 786
Morton, R. A., Heilbron, I. M. and Thompson, A. Spectrographic data concerning vitamin A and liver oils 20

MORTON, R. A., *see also* COWARD, K. H., GILLAM, A. E., HEILBRON, I. M. and LOVERN, J. A.
Muscle, frog, isolated, carbohydrate content of (Kerly) 671
Muscle metabolism, occurrence of pyruvic acid and methylglyoxal in (Case and Cook) 1319
Muscle, "non-irritable" (McCullagh and Case) 1220

NARAIN, R., *see* HULME, A. C.
NARAYANA, B., *see* REID, C.
NARAYANAMURTI, D. and RAMASWAMI, C. V. The nature of tyrosinase 749
Narcotics, effect of, on dehydrogenases (Sen) 840
Nematospora gossypii, accessory factor necessary for the growth of (Buston and Pramanik) 1656, 1671
Nephritis, chlorides and inorganic constituents of serum and cerebrospinal fluid in (Linder and Carmichael) 1090
NICHOLSON, W. N., NIERENSTEIN, M., POOL, J. C. and PRICE, N. V. The action of tannase on gallotannin 752
NIERENSTEIN, M., *see* NICHOLSON, W. N.
NILSSON, R., *see* v. EULER, H.
Nitrates, reduction of, by *B. coli* (Stickland) 1543
Nitrogen balance in vitamin B_2 deficiency in the rat (Kon) 482
Nitrogen, dicarboxylic acid-, of proteins (Damodaran) 2123
Nitrogen, distribution of, in urine during increased catabolism (Cuthbertson) 236
Nitrogen excretion, effect of insulin on (Goldblatt and Ellis) 221
Nitrogen immobilisation during decomposition of plant materials (Richards and Norman) 1769
Nitrogen immobilisation by straw, effect of p_H on (Norman) 1779
Nitrogen, liberation of elementary, by bacteria (Barritt) 1965
Nitrogen metabolism of the lupin seedling (McKie) 2181
Nitrogen, urinary output of, in relation to cyclic changes in glycogen content of liver and muscles of rats and mice (Ågren, Wilander and Jorpes) 777
"Non-irritable" muscle (McCullagh and Case) 1220
NORMAN, A. G. Studies on the gums. II. Tragacanthin—the soluble constituent of gum tragacanth 200
NORMAN, A. G. The biological decomposition of plant materials. VI. The effect of hydrogen ion concentration on the rate of immobilisation of nitrogen by straw 1779
NORMAN, A. G., *see also* RICHARDS, E. H.
Nutrition, copper in animal (Cunningham) 1267
Nutrition, experiments on (Plimmer, Raymond and Lowndes) 1788

Obituary notice of James Lorrain Smith 1849
O'DWYER, M. H. The hemicelluloses of the wood of English oak. I. The effect of the drying of the wood on the yields and composition of hemicellulose A 2017
Oil development in the seed of a growing plant (Eyre) 1902
Oleic acid, oxidation of, by hydrogen peroxide (Smedley-MacLean and Pearce) 1252
Orange, vitamin C in (Bracewell and Zilva) 1081
OSBORN, T. W. B. A study of the factors influencing the concentration of complement in the blood 2136
Ossification, heterotopic, produced by phosphatase activity of transplants of epithelium of urinary bladder (Huggins) 728
Ovalbumin and gelatin, electrophoretic velocities of, in their mixtures (Prideaux and Howitt) 391
Ovary, fowl's, growth and chemical composition of (Romanoff) 994
OVERHOFF, J., *see* BRUINS, H. R.
Oxidation by *B. coli communis*, effect of hydrogen ion concentration on (Cook and Alcock) 523
Oxidation of oleic acid by hydrogen peroxide (Smedley-MacLean and Pearce) 1252
Oxidation of sulphydryl compounds by hydrogen peroxide (Pirie) 1565
Oxidation-reduction potentials, behaviour of indifferent electrodes in determination of, in presence of hydrogen (Lepper and Martin) 45
Oxidation-reduction potentials of *B. coli communis* cultures, effect of bacteriophage on (Hewitt) 1641
Oxidation-reduction potentials of *B. dysenteriae* cultures, effect of bacteriophage on (Hewitt) 1447
Oxidation-reduction potentials of haemolytic streptococci, effect of catalase on (Hewitt) 858
Oxidation-reduction potentials of *M. lysodeikticus* cultures, effect of lysozyme on (Hewitt) 1452
Oxidation-reduction potentials of pneumo coccus cultures, effect of catalase on (Hewitt) 169
Oxidation-reduction potentials of *Staphylococcus* cultures, effect of bacteriophage on (Hewitt) 2068
Oxidations, biological, in succinic acid series (Quastel and Wheatley) 117
Oxidations, effect of dyestuffs on (Quastel and Wheatley) 629
Oxido-reductions, activation of, by co-zymase (v. Euler and Nilsson) 2168
Oxycoccus macrocarpus Pers., anthocyanin of (Grove and Robinson) 1706
OYA, C., *see* SINODA, O.

PACE, J. The effect of heat upon the activating efficiency of enterokinase 1
PACE, J. The inactivation of trypsin-kinase by heat and the effect of added protein thereon 422
PACE, J. The heat-inactivation of the proteinase of the pancreas 1485
Paraffins, ketones and secondary alcohols, synthesis and crystal spacings of long-chain (Piper, Chibnall, Hopkins, Pollard, Smith and Williams) 2072
PATTERSON, L. M. B. The effects of cyanide and some other salts on fermentation by yeast preparations 1593

PEARCE, M. S. B., *see* SMEDLEY-MACLEAN, I.
Peas, loss of glucose from dried, on soaking (Clifford) 1999
Penicillium griseo-fulvum, production of hydroxymethylbenzoic acid from glucose by action of (Anslow and Raistrick) 39
Penicillium puberulum Bainier, isolation of ergosterol from (Birkinshaw, Callow and Fischmann) 1977
Pepsin, enzymic synthesis of proteins by (Cuthbertson and Tompsett) 2004
Peroxidase action, kinetics of (Mann) 918
PETERS, R. A., *see* GAVRILESCU, N.
Phloridzin, effect of, on glycogen distribution in the rat (Lawrence and McCance) 570
Phosphatase activity of transplants of epithelium of urinary bladder (Huggins) 728
Phosphatases, blood- (Roche) 1724
Phosphate buffer mixtures, vapour pressure depressions of, at 20·3° (Grollman) 166
Phosphatide and cholesterol contents of normal and malignant human tissues (Jowett) 1991
Phosphatides and cholesterol, determination of small amounts of (Jowett and Lawson) 1981
Phosphorus metabolism of guinea-pigs in scurvy (Humphreys and Zilva) 579
Pigmentation of the liver of the monk (or angler) fish (Lovern and Morton) 1336
PIPER, S. H., CHIBNALL, A. C., HOPKINS, S. J., POLLARD, A., SMITH, J. A. B. and WILLIAMS, E. F. Synthesis and crystal spacings of certain long-chain paraffins, ketones and secondary alcohols 2072
PIPER, S. H., *see also* CHIBNALL, A. C. and POLLARD, A.
PIRIE, N. W. The cuprous derivatives of some sulphydryl compounds 614
PIRIE, N. W. The oxidation of sulphydryl compounds by hydrogen peroxide. I. Catalysis of oxidation of cysteine and glutathione by iron and copper 1565
PIRIE, N. W., *see also* HELE, T. S.
Pituitrin, effect of, on glycogen distribution in the rat (Lawrence and McCance) 570
Plant materials, biological decomposition of (Richards and Norman) 1769, (Norman) 1779
Plant, oil development in seed of growing (Eyre) 1902
Plastein formation (Cuthbertson and Tompsett) 2004
PLEASS, W. B. The absorption of water by gelatin. VII. The influence of temperature on swelling in acid solutions and the combination of gelatin with hydrochloric, nitric or sulphuric acid 1943
PLIMMER, R. H. A., RAYMOND, W. H. and LOWNDES, J. Experiments on nutrition. X. Comparative vitamin B_1 values of foodstuffs. Cereals II 691
PLIMMER, R. H. A., RAYMOND, W. H. and LOWNDES, J. Experiments on nutrition. XI. Comparative vitamin B_1 values of foodstuffs. Fruits and vegetables 1788
Pneumococcus cultures, oxidation-reduction potentials of, effect of catalase on (Hewitt) 169
POLLARD, A., CHIBNALL, A. C. and PIPER, S. H. The wax constituents of forage grasses. I. Cocksfoot and perennial ryegrass 2111
POLLARD, A., *see also* CHIBNALL, A. C. and PIPER, S. H.
POOL, J. C., *see* NICHOLSON, W. N.
POPE, C. G. A method for determining aluminium in certain aluminium protein compounds 1949
Potato tuber, hydrogen ion concentration of (Robertson and Smith) 763
Potatoes, blackening of, after cooking (Tinkler) 773
PRAMANIK, B. N., *see* BUSTON, H. W.
PREECE, I. A. Studies on hemicelluloses. IV. The proximate analysis of box-wood, and the nature of its furfuraldehyde-yielding constituents 1304
PRENTICE, J. H., *see* CAPPER, N. S.
PRICE, H. I. Autolysis in malignant and normal rabbit tissues 1491
PRICE, N. V., *see* NICHOLSON, W. N.
PRIDEAUX, E. B. R. and HOWITT, F. O. The electrophoretic velocities of gelatin and ovalbumin in different concentrations of their mixtures and the effect of ultra-violet irradiation 391
Proteases, bacterial, formation of, in synthetic media (Haines) 1851
Protein, alcohol-soluble from ragi (*Eleusine coracana*) (Damodaran) 2123
Proteinase of green malt, effect of, on egg-albumin, caseinogen, edestin and fibrin (Hopkins and Kelly) 256
Proteinase of the pancreas, heat-inactivation of (Pace) 1485
Proteins, alcohol-soluble, from milk products (Allen) 1045
Proteins and protein derivatives, influence of, on enzymic hydrolysis of starch by malt diastase (Filipowicz) 1874
Proteins, determination of cystine in (Tompsett) 2014
Proteins, dicarboxylic acid-nitrogen of (Damodaran) 2123
Proteins, effect of p_H on precipitating and protective action of mixtures of, on colloidal gold and gum benzoin (Hopkins, Kermack and Spragg) 829, 1860
Proteins, effect of salts on isoelectric point of (Przyłęcki) 713; (Przyłęcki and Giedroyć) 1734
Proteins, enzymic synthesis of (Cuthbertson and Tompsett) 2004
Proteins of breads, biological values of (Kon and Markuze) 1476
Proteins, precipitating and protective actions of, on colloidal gold and gum benzoin, effect of hydrogen ion concentration on (Hopkins, Kermack and Spragg) 829, 1860
Proteins, serum, carbohydrate complex of (Rimington) 1062
Proteolytic enzymes of green malt (Hopkins and Kelly) 256
PRZYŁĘCKI, S. J. Effect of addition of salts on the isoelectric point of proteins 713
PRZYŁĘCKI, S. J. and GIEDROYĆ, W. Effect of addition of salts on the isoelectric point of proteins II. 1734
PRZYŁĘCKI, S. J., *see also* GIEDROYĆ, G.

Pyruvic acid, occurrence of, in muscle metabolism (Case and Cook) 1319

QUASTEL, J. H. The action of dyestuffs on enzymes. II. Fumarase 898
QUASTEL, J. H. Trypanocidal action and toxicity to enzymes 1121
QUASTEL, J. H. and WHEATLEY, A. H. M. Biological oxidations in the succinic acid series 117
QUASTEL, J. H. and WHEATLEY, A. H. M. The action of dyestuffs on enzymes. I. Dyestuffs and oxidations 629
Quills, porcupine, sulphur distribution in component structures of (Bekker and King) 1077

Radiation, ultra-violet, effect of, on electrophoretic velocities of gelatin and ovalbumin in their mixtures (Prideaux and Howitt) 391
Radiation, ultra-violet, permeability of human epidermis to (Lucas) 57
RAISTRICK, H., *see* COYNE, F. P. and ANSLOW, W. K.
RAMAGE, H., *see* SHELDON, J. H.
RAMASWAMI, C. V., *see* NARAYANAMURTI, D.
RANDALL, S. S., *see* HARINGTON, C. R.
Rat, normal nutrition of, factors necessary for (Guha) 960
Rat, self-selection of food constituents by the (Kon) 473
RAYMOND, W. H., *see* PLIMMER, R. H. A.
Reductase, reactivation of, in washed yeast preparations (Harden and Macfarlane) 818
REERINK, E. H. and VAN WIJK, A. The vitamin D problem. II. Optical rotation of vitamin D 1001
REEVES, H. G. and RENBOM, E. T. Note on dihydroxyacetone 412
REID, C. and NARAYANA, B. Studies in blood glycolysis. Preliminary observations 339
Renal threshold for glucose, determination of (Himsworth) 1128
RENBOM, E. T., *see* REEVES, H. G.
Respiratory catalysts of *B. coli*, relationship between (Cook, Haldane and Mapson) 534
Respiratory quotient of tissues, measurement of (Dickens and Šimer) 973, 985
"Resting" bacteria (Sandiford and Wooldridge) 2172
RICHARDS, E. H. and NORMAN, A. G. The biological decomposition of plant materials. V. Some factors determining the quantity of nitrogen immobilised during decomposition 1769
RICHARDSON, F., *see* MCFARLANE, W. D.
RICHARDSON, G. M., *see* HARINGTON, C. R.
RIMINGTON, C. The relation between cystine yield and total sulphur in kemp and outercoat animal fibres 71
RIMINGTON, C. The carbohydrate complex of the serum-proteins. II. Improved method for isolation and re-determination of structure. Isolation of glucosaminodimannose from proteins of ox blood 1062
RIMINGTON, C., *see also* BARRITT, J. and STEWART, A. M.
ROBERTSON, I. M. and SMITH, A. M. A study of the hydrogen ion concentration of the potato tuber 763
ROBINSON, G. M. and ROBINSON, R. A survey of anthocyanins. I 1687
ROBINSON, R., *see* GROVE, K. E. and ROBINSON, G. M.
ROBISON, R. and KING, E. J. Hexosemonophosphoric esters 323
ROBSON, W., *see* LAMB, J.
ROCHE, J. Blood-phosphatases 1724
ROMANOFF, A. L. Growth and chemical composition of functioning fowl's ovary (*Gallus domesticus*) 994
ROSCOE, M. H. The distribution of the vitamin B complex. II. Root vegetables 1205
ROSCOE, M. H. The distribution of the vitamin B complex. III. Fruits 2050
ROSCOE, M. H. The effects of coprophagy in rats deprived of the vitamin B complex 2056
ROSENHEIM, O. and CALLOW, R. K. Colour reactions of sterols with nitric acid 74
ROTTER, D. L., *see* HYND, A.

Saccharase, significance of Michaelis constant for (Briggs) 1801
SALT, H. B. The colorimetric determination of uric acid in urine 1720
SALT, H. B. The ergothioneine content of the blood in health and disease 1712
Salts, effect of addition of, on the p_H of solutions of ampholytes (Giedroyć and Przyłęcki) 465
Salts, effect of, on isoelectric point of proteins (Przyłęcki) 713
SANDIFORD, B. R. and WOOLDRIDGE, W. R. "Resting" bacteria 2172
Scaly tail condition in rats, relation of fat-free diet to (Hume and Smith) 300
SCHIØDT, E. On the quantity of free water in the red blood corpuscles 8
SCHULTZER, P. Investigations on the determination of vitamin D. Comparison between the preventive and the curative methods 1745
Scurvy, calcium and phosphorus metabolism of guinea-pigs in (Humphreys and Zilva) 579
SELBY, D. L., *see* HARDING, V. J.
SEN, K. C. The effect of narcotics on some dehydrogenases 849
SEN, K. C. The sensitisation of red blood corpuscles in haemolysis 1244
SEN, K. C., *see also* MAGEE, H. E.
Serum, action of, on trypsin (Fine) 647
Serum, chlorides and inorganic constituents of, in nephritis and acidosis (Linder and Carmichael) 1090
SHELDON, J. H. and RAMAGE, H. A spectrographic analysis of human tissues 1608
SHIPP, H. L., *see* MCCANCE, R. A.
DA SILVA, G. A. The determination of tryptophan and tyrosine 1634
ŠIMER, F., *see* DICKENS, F.
SINODA, O., KODERA, S. and OYA, C. The chemistry of cooking. I. The chemical changes of carbohydrates in the sweet potato according to various methods of cooking 1973
SLEIGHTHOLME, J. J., *see* HILDITCH, T. P.

SMEDLEY-MACLEAN, I. and PEARCE, M. S. B. The oxidation of oleic acid by means of hydrogen peroxide with and without the addition of copper sulphate: a possible analogy with its oxidation *in vivo* 1252
SMEDLEY-MACLEAN, I., *see also* COLLISON, D. L.
SMITH, A. M., *see* ROBERTSON, I. M.
SMITH, H. H., *see* HUME, E. M.
SMITH, J. A. B., *see* CHIBNALL, A. C. and PIPER, S. H.
Sodium, determination of, colorimetrically (McCance and Shipp) 449
Sodium, micro-determination of, in biological materials (McCance and Shipp) 1845
Softwood, drying of a (Campbell and Booth) 756
Spectrographic analysis of human tissues (Sheldon and Ramage) 1608
Spermine in human tissues (Harrison) 1885
SPIERS, M. A., *see* HIMWICH, H. E.
Spinach, lipoid matter extracted from (Collison and Smedley-MacLean) 606
SPRAGG, W. T., *see* HOPKINS, R. H.
Spruce wood, products of decay of, rotted by *Merulius lachrymans* (Barton-Wright and Boswell) 494
Staphylococcus cultures, oxidation-reduction potentials of, effect of bacteriophage on (Hewitt) 2068
Starch, estimation of products of hydrolysis of, by taka-diastase (Widdowson) 863
Starvation, effect of, on glycogen distribution in the rat (Lawrence and McCance) 570
STEDMAN, E. and STEDMAN, E. Studies on the relationship between chemical constitution and physiological action. III. The inhibitory action of certain synthetic urethanes on the activity of liver esterase 1147
STEINER, A., *see* KRAMER, H.
STENT, H. B., *see* WALKER, T. K.
STEPHENSON, M. and STICKLAND, L. H. Hydrogenase, a bacterial enzyme activating molecular hydrogen. I. The properties of the enzyme 205
STEPHENSON, M. and STICKLAND, L. H. Hydrogenase. II. The reduction of sulphate to sulphide by molecular hydrogen 215
Sterols, colour reactions of, with nitric acid (Rosenheim and Callow) 74
STEWART, A. M. and RIMINGTON, C. The basic amino-acids of wool 2189
STEWART, C. P., GADDIE, R. and DUNLOP, D. M. Fat metabolism in muscular exercise 733
STICKLAND, L. H. The reduction of nitrates by *B. coli* 1543
STICKLAND, L. H., *see also* STEPHENSON, M.
Stout as an addition to white wheat flour or fish meal as a diet for pigs (Blissett and Golding) 349
Streptococci, haemolytic, effect of catalase on oxidation-reduction potentials of (Hewitt) 858
SUBRAMANIAM, V., *see* WALKER, T. K.
Succinic acid series, biological oxidations in (Quastel and Wheatley) 117
Sucrose, oxidation of, in percolating filters (Jenkins) 147
Sucrose solutions, dilute, biological filtration of (Barritt) 1419
Sugar alcohols, occurrence of, in marine algae (Haas and Hill) 1470
Sugar, fermentable, in normal urine (Harding and Selby) 1815
Sugar solutions, hydrolysis of concentrated, by invertase (Auden and Dawson) 1909
Sugars, reducing, determination of, iodimetrically, in the apple (Archbold and Widdowson) 101
Sugars, reducing, ferricyanide method for determination of (Hulme and Narain) 1051
Sugars, reducing, mixed, determination of small quantities of (Widdowson) 863
Sulphate, enzymic reduction of, by molecular hydrogen (Stephenson and Stickland) 215
Sulphate, inorganic, content and determination of, in the blood (Cuthbertson and Tompsett) 1237
Sulphate, inorganic, determination of, in human blood-plasma (Cope) 1183
Sulphates, ethereal, synthesis in the dog (Hele) 1736
Sulphur, distribution of, in urine during increased catabolism (Cuthbertson) 236
Sulphur metabolism of the dog (Hele) 1736
Sulphur metabolism of the dog (Hele and Pirie) 1095
Sulphydryl compounds, cuprous derivatives of (Pirie) 614
Sulphydryl compounds, oxidation of, by hydrogen peroxide (Pirie) 1565
Sunlight, influence of, on milk (Whitehead) 1647

Taka-diastase, hydrolysis of starch by (Widdowson) 363
Tallows, beef, glyceride structure of (Banks and Hilditch) 1168
Tannase, action of, on gallotannin (Nicholson, Nierenstein, Pool and Price) 752
Tetany, metabolism in (Morris, Watson and Morris) 786
Thermometer, a differential resistance, for biological use (McCullagh and Case) 1213
THOMPSON, A., *see* MORTON, R. A.
Thyroid, effect of, on glycogen distribution in the rat (Lawrence and McCance) 570
Thyroid gland, isolation of *d*-3 : 5-diiodotyrosine from (Harington and Randall) 1032
Thyroxine, effect of arginine on body weight of tumour-bearing mice injected with (Gilroy) 414
Timber, chemical aspect of drying of (Campbell and Booth) 756
TINKLER, C. K. The blackening of potatoes after cooking 773
Tissues, human, spectrographic analysis of (Sheldon and Ramage) 1608
Tissues, human, spermine in (Harrison) 1885
Tissues, normal and malignant human, phosphatide and cholesterol contents of (Jowett) 1991
Tissues, rabbit, autolysis in malignant and normal (Price) 1491
TOMPSETT, S. L. A note on the determination of cystine in proteins by the method of Folin and Marenzi 2014

TOMPSETT, S. L., *see also* CUTHBERTSON, D. P.
Torulin, function of. An *in vitro* effect (Gavrilescu and Peters) 2150
Tragacanthin (Norman) 200
Trypanocidal action and toxicity to enzymes (Quastel) 1121
Trypsin, action of serum on (Fine) 647
Trypsin-kinase, inactivation of, by heat (Pace) 422
Tryptophan, determination of (da Silva) 1634
Tumour-bearing mice injected with thyroxine, effect of arginine on the body weight of (Gilroy) 414
Tumour tissue, metabolism of (Dickens and Šimer) 973, 985
TURNER, K. The fatty acids of the cat's kidney. I 49
Tyrosinase (Narayanamurti and Ramaswami) 749
Tyrosine, determination of (da Silva) 1634

Ultra-violet absorption of liver oils (Gillam and Morton) 1346
Urea in urine, method for direct determination of (Cole) 1653
Urease-urea-gelatin system (Freiberger) 705
Urethanes, inhibitory action of, on activity of liver esterase (Stedman and Stedman) 1147
Uric acid, colorimetric determination of, in urine (Salt) 1720
Urine, "ether reaction" of (Boyd) 812
Urine, normal, fermentable sugar in (Harding and Selby) 1815

VAHLQUIST, B. Blood-phosphorus in fat absorption 1628
Vegetables, root, distribution of vitamin B complex in (Roscoe) 1205
Vitamin A and carotene (Moore) 2131
Vitamin A content of fish meal and meat meal (McFarlane, Graham and Richardson) 358
Vitamin A, conversion of carotene into, by fowl (Capper, McKibbin and Prentice) 265
Vitamin A, determination of, in cod-liver oils (Coward, Dyer, Morton and Gaddum) 1102
Vitamin A, distribution of, in the body of the rat (Moore) 275
Vitamin A, effect of addition of, to a rachitogenic diet (Bacharach, Allchorne and Hazley) 639
Vitamin A, molecular weight of (Bruins, Overhoff and Wolff) 430
Vitamin A reserves of the rat, effect on, of vitamin B deficiency (Dann and Moore) 914
Vitamin A, specificity in tests for (Heilbron, Gillam and Morton) 1352
Vitamin A, spectrographic data concerning (Morton, Heilbron and Thompson) 20
Vitamin A, spectrographic data of natural fats and their fatty acids in relation to (Gillam, Heilbron, Hilditch and Morton) 30
Vitamin B complex, distribution of (Roscoe) 1205
Vitamin B complex, distribution of, in fruits (Roscoe) 2050
Vitamin B complex, effects of coprophagy in rats deprived of (Roscoe) 2056
Vitamin B deficiency, biochemical lesions in (Gavrilescu and Peters) 1397
Vitamin B deficiency, effect of, on vitamin A reserves of the rat (Dann and Moore) 914
Vitamin B_1, chemical nature of, from evidence afforded by its electrical transference (Birch and Guha) 1391
Vitamin B_1 concentrates from yeast, preparation and behaviour of (Guha) 931
Vitamin B_1 deficiency, blood-sugar in (Bell) 1755
Vitamin B_1 physiological function of (Guha) 1367
Vitamin B_1 values of cereals (Plimmer, Raymond and Lowndes) 691
Vitamin B_1 values of fruits and vegetables (Plimmer, Raymond and Lowndes) 1788
Vitamin B_2 deficiency in the rat, nitrogen balance in (Kon) 482
Vitamin B_2, sources, stability and chemistry of (Guha) 945
Vitamin C, estimation of (Key and Elphick) 888
Vitamin C in orange and grape fruit (Bracewell and Zilva) 1081
Vitamin concentrates, an *in vitro* effect of antineuritic (Gavrilescu and Peters) 2150
Vitamin D content of fish meal and meat meal (McFarlane, Graham and Richardson) 358
Vitamin D, determination of (Schultzer) 1745
Vitamin D, fundamental nature of action of (McGowan, Cunningham and Auchinachie) 1295
Vitamin D, mode of action of (Harris and Innes) 367
Vitamin D, occurrence of, in lampreys (Callow and Fischmann) 1464
Vitamin D, optical rotation of (Reerink and van Wijk) 1001
Vitamin tests, caseinogen for (Coward, Key, Dyer and Morgan) 551
VOGT-MØLLER, P. Is avitaminosis B_1 an intoxication by methylglyoxal? Glyoxalase-co-enzyme ratio in experimental beriberi 418
VOGT-MØLLER, P. On the glyoxalase-co-enzyme ratio in liver tissue in inanition 1540

WALKER, T., SUBRAMANIAM, V., STENT, H. B. and BUTTERWORTH, J. A study of the mechanism of the degradation of citric acid by *B. pyocyaneus* (*Pseudomonas pyocyanea*). II. Action of *B. pyocyaneus* on succinic acid 129
WALLACE, T., *see* BRACEWELL, M. F.
WATCHORN, E. and HOLMES, B. E. Studies in the metabolism of tissues growing *in vitro*. IV. Effect of fructose, galactose and xylose upon the ammonia and urea production of embryo kidney tissue 843
WATSON, A. McL., *see* MORRIS, S.
Wax constituents of apple cuticle (Chibnall, Piper, Pollard, Smith and Williams) 2095
Wax constituents of forage grasses (Pollard, Chibnall and Piper) 2111
WEICHSELBAUM, T. E., *see* BARGER, G.
WEST, C., *see* BRACEWELL, M. F.

Wheat flour, white, inadequacy of, for prolonged growth of pigs (Blissett and Golding) 349
WHEATLEY, A. H. M., *see* QUASTEL, J. H.
White rots of wood, chemistry of, II (Campbell) 2023
WHITEHEAD, H. R. The influence of sunlight on milk 1647
WIDDOWSON, E. M. A method for the determination of small quantities of mixed reducing sugars and its application to the estimation of the products of hydrolysis of starch by taka-diastase 863
WIDDOWSON, E. M., *see also* ARCHBOLD, H. K.
VAN WIJK, A., *see* REERINK, E. H.
WILANDER, O., *see* ÅGREN, G.
WILLIAMS, E. F., *see* CHIBNALL, A. C. and PIPER, S. H.
WOLFF, L. K., *see* BRUINS, H. R.
Wood, chemistry of white rots of (Campbell) 2023
Wood of English oak, hemicelluloses of (O'Dwyer) 2017
Wool and porcupine quills, sulphur distribution in component structures of (Bekker and King) 1077
Wool, basic amino-acids of (Stewart and Rimington) 2189
Wool, black-face, sulphur-free nature of medulla in (Barritt and King) 1075
WOOLDRIDGE, W. R., *see* SANDIFORD, B. R.
WOOLF, B. The addition compound theory of enzyme action 342

Xylose, effect of, on ammonia and urea production of embryo kidney tissue *in vitro* (Watchorn and Holmes) 843

Yeast, as an addition to white wheat flour or fish meal as a diet for pigs (Blissett and Golding) 349
Yeast, preparation and behaviour of vitamin B_1 concentrates from (Guha) 931
Yeast preparations, effects of cyanide and other salts on fermentation by (Patterson) 1593
Yeasts, brewer's and Sauterne, selective fermentation of glucose and fructose by (Hopkins) 245

ZILVA, S. S. A note on the reprecipitation of the antiscorbutic factor from decitrated lemon juice 594
ZILVA, S. S., *see also* BRACEWELL, M. F. and HUMPHREYS, F. E.
Zymin, induction period of, effect of arsenate on (Macfarlane) 822

CAMBRIDGE: PRINTED BY W. LEWIS, M.A., AT THE UNIVERSITY PRESS

PRICES OF BACK NUMBERS AND VOLUMES.

In Paper covers.

Volumes I–VI.	31*s.* 6*d.* net per vol.	Quotations can be given by the publishers for bound copies of back numbers.
Volumes VII–X.	(*Out of print.*)	
Index of Authors and Subjects in Volumes I–X.	5*s.* 3*d.* net. (By post, 5*s.* 5*d.*)	
Volumes XI–XVI.	(*Out of print.*)	
Volumes XVII–XVIII.	60*s.* net per vol.	
Volume XIX.	(*Out of print.*)	
Volumes XX–XXIV.	60*s.* net per vol.	
Index of Authors and Subjects in Volumes XI–XX.	10*s.* net. (By post, 10*s.* 4*d.*)	

Claims for Journals lost in Post will not be entertained more than 3 months after the date of posting of the Journal.

For Notice to Contributors see page 2 of Wrapper

PRINTED IN GREAT BRITAIN BY W. LEWIS, M.A., AT THE UNIVERSITY PRESS, CAMBRIDGE

C37

0585
1 32E
2 5E3
3 32E
4 283
5 582
6 E38